ELETROPHORETIC STUDIES OF SERUM PROTEIN AND EYE LENS PROTEIN OF SOME FRESHWATER FISHES OF ALIGARH

ABSTRACT

Thesis submitted
to the Aligarh Muslim University, Aligarh

By

SEIKH AMJED ALI, M. Sc., M. Phil. (Alig.)

in candidature for the degree of

DOCTOR OF PHILOSOPHY

in

ZOOLOGY

DEPARTMENT OF ZOOLOGY
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ELECTROPHORETIC STUDIES OF SERUM PROTEIN AND EYE LENS PROTEIN OF SOME FRESHWATER FISHES OF ALIGARH.

ABSTRACT

The eye lens proteins of 26 species, belonging to 8 families, and serum proteins of 10 species were separated by starch gel electrophoresis.

The soluble eye lens proteins of each species were investigated in detail and compared with other related species. Evidence is presented for the virtual constancy and species specific nature of eye lens protein patterns. On the basis of the distribution of the soluble protein components phylogenetic relationships were established among the species of the same genus as well as with other species. Phylogenetic relationships based on biochemical characters supported the morphologically based relationship in the genus *Mystus* and *Ophicephalus*.

Remarkable species differences were shown by slow migrating low molecular weight proteins, whereas the farthest migrating high molecular weight proteins on the cathodal side were much more stable not only among the species of the same genus, but also in the same family and even in the same order.

Lens protein polymorphism was not observed in any species studied, except *Labeo bata*. Two distinct patterns were found

belonging to fishes of two localities. Differences in lens protein patterns of mature and immature *Cirrhina mrigala* were also observed but no such difference was noted in other fish species. Sex, size and environment have no effect in the eye lens electropherograms. Both right and left eye lenses produced identical protein patterns.

The soluble proteins of cortex, nucleus and whole eye lens of two closely related species *Ophicephalus punctatus* and *Ophicephalus gachua*, were examined by starch gel electrophoresis. Whole eye lens and cortical protein patterns were found to be more or less similar but distinctly different patterns were obtained for nucleus. The nucleus proteins were found to resist denaturation as compared to cortex and whole eye lens proteins under physical stress.

A suitable saline solution was sought which would solubilize all classes of lens proteins. Starch gel electrophoresis was performed on the lens extracts of distilled water and 26 different concentrations of sodium chloride solutions. High concentrations of salt solutions produced 'salt effects' on electrophoretic patterns. Low concentrations of salt solutions produced maximum number of protein zones. 0.015 to 0.020 g% salt solutions were found to be most efficient to solubilise all kinds of proteins of all layers (cortex and nucleus) of lenses.

Proteins of *O. punctatus* were found to be more heat stable than that of *O. striatus*. Lens protein patterns of heated extracts for different time intervals show a remarkable change in the lens proteins. Variations were more in low molecular weight proteins which were less stable than the high molecular weight proteins.

A remarkable parallelism was observed in the serum protein patterns of closely related species. Intraspecific variations were common in most of the species. Various factors, such as genetic, physiological, ecological were found to be responsible for these changes of patterns. Examples of species specificity, ontogenetic variation, geographic variation and various polymorphisms are described, together with problems concerning with the segregation of proteins. Variations in proteins patterns were not found to be associated with sex, except in *Clarias batrachus*. 75 days of starvation were required to change the serum protein patterns in *Heteropneustes fossilis*.

One lipoprotein fraction was noted darkly stained in female fish during spawning time. Polymorphism was not observed in transferrin fractions.

Serum esterase patterns of four species of *Ophiocephalus* were found to be monomorphic. Polymorphism of serum esterase pattern was not observed in any of the species of *Ophiocephalus*.

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Ref. No.

Date October 9, 1974.

DECLARATION

This is to certify that Mr. Seikh Amjed Ali has completed his research work under the supervision of Dr. A. Qayyum Siddiqui. But the work could not be submitted in the form of a thesis for the award of Ph.D. degree under Dr. A. Qayyum Siddiqui, as he left Aligarh to take an assignment at Kenyatta University, Nairobi, Kenya. Now the thesis is being submitted under my direct supervision. The observational work recorded in this thesis was carried out by the candidate himself and I consider it a good piece of work with a considerable amount of addition in the existing knowledge on chemo-taxonomy of fishes. He is allowed to submit his thesis for the award of Ph.D. degree in Zoology of the Aligarh Muslim University, Aligarh.

(Asif Ali Khan)
Ph.D.

CONTENTS

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It gives a great pleasure to me to acknowledge my indebtedness to Dr. A. Qayyum Siddiqui, under whose supervision the present work was carried out and completed. However, due to some technical difficulties the thesis could not be submitted under him as he took an assignment at Kenyatta University, Nairobi, Kenya. The thesis is being submitted under Dr. Asif Ali Khan, to whom I am extremely thankful for his interest in the work and encouragements throughout the whole period of research.

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Thanks are also due to all of my friends and research scholars of the Fisheries Research Laboratory who helped me during the course of this work.

GENERAL INTRODUCTION AND HISTORICAL RESUME

Taxonomy is one of the most classical branch of science gaining an ever increasing attention in various forms of research. The early classification based on morphological characters has recently been modified and some new techniques have been evolved. To solve some important unsolved problems, the biochemical systematics, which is based on the specificity of chemical constituents, offer a more stable basis for taxonomic research. The application of biochemical techniques to animal taxonomy and systematics has opened a new era and has become increasingly widespread (Sibley, 1967, 1970; Tsuyuki et al., 1968). It has been found to be very useful in the solution of important problems of phylogeny, ontogeny, population differences and taxonomical classification of different categories (Manwell and Baker, 1963; Hyman and Westin, 1968, 1969).

The migration of a particle in an electric field is necessary since it possesses a charge. The proteins and enzymes are charged groups and therefore, it can be analysed electrophoretically which is based on their differential migration of ions in an electric field. The velocity (V) in an electric field (E) with charge (Q) in a nonconducting viscous fluid is $V = \frac{Q}{f} E$. The frictional coefficient (f) is defined by Stokes law, $f = 6\pi nr$, where n is the viscosity of the liquid and r is the particle radius. Therefore, the mobilities of proteins have been found to be proportional to their charge (Brown and Timasheff, 1959). On this basis different electrophoretical methods have been used for determining the isoelectric points and also for the fractionation of protein mixtures. The isoelectric point of a protein is defined as the pH at which the protein does not move in an electric field. The measurement of protein mobilities and the isoelectric point under specified conditions has also been useful in classification and characterization.

In species where morphological similarity is great, the electrophoretic separation and structural studies of proteins may provide to the systematists and taxonomists an additional taxonomic character at the molecular level of organization. There are also some taxonomic problems which are very difficult at the subspecies level, since most morphological characters are modified according to environment. Therefore, biochemical analysis of proteins may add considerably to the knowledge of genetic changes and thus may be a good source to solve the problems of phylogeny. Nuttall (1901) was one of the first who tried to determine the significance of species specificity in the properties of proteins by means of immunological techniques. Due to rapid advancement in the field of protein chemistry it is now well known that Deoxyribose Nucleic Acid (DNA) is responsible for genetic information which constitute the genes of the chromosome, where direct translation is made through various stages of coding by different forms of nucleic acids into protein molecules (Crick, 1963; Marmur et al., 1963; Nirenberg et al., 1963; Yanofsky et al., 1963). Sibley (1960) stated that the principal morphological units of the animal body are protein molecules which are a good source of genetic and phylogenetic information. Species specific protein variations consequently occur by the action of a number of different genes. Although the structure of proteins are well known and provide useful genetic differentiation but the methodology is not yet adaptable for quick comparisons of large number of proteins on a wide scale. With the recent development of various electrophoretical methods for the separation of proteins in combination with histochemical staining procedures, one is capable of demonstrating different proteins as coloured bands in the colourless medium. The study of protein variations may inform the hereditary differences between the populations and the species by these techniques. High resolution starch gel electrophoretic studies can be used to advantage for studying comparative electrophoretic mobilities of proteins. The electrophoretic studies in taxonomical work are no doubt useful but some factors are sometimes complicated which must be kept in mind during the course of studies as follows : (I) problem of analogous and homologous genes and (II) occurrence of ontogenetic variations in the species. It has been found in many species that during the embryonic development the protein patterns are completely changed in their phenotypic appearance. Radical changes of patterns were obtained in various stages of growth until it attained the sexual maturity. Another problem is whether homologous or analogous genes are responsible for identical phenotypes.

There are also certain inherent limitations in the electrophoretic analysis of proteins which must be recognised. (a) In any one species protein electrophorograms must be virtually identical excepting genetically linked polymorphisms and (b) identical mobilities of proteins tend to become less clear at higher level of organization. It is known that various tissues of the body are composed of almost invariable proteins. Since there are many proteins which are not only species specific but organ specific. Therefore the investigation of the protein systems may provide good source for the study of inter and intraspecific variations, ontogeny, general systematics and phylogeny.

Although various methods have been applied successfully by many authors to prove the affinities and differences among and within the species, the study of proteins of living systems is found to be more remarkable. It was Anfinsen (1959) who first pointed out the interrelationship between the protein and gene structure. Sibley (1960) examined by paper electrophoresis over 5000 eggs white proteins of aves for classification purposes. Dessauer and Fox (1956) reported 1200 plasma patterns from 300 specimens of at least 100 species of amphibians and reptiles. Moreover, the evolutionary events and the organisms have been the result of changes in the genetic message.

The application of biochemical systematics to fishes is the most stable source than the earlier existing methods, based on morphological characters. The population study of fishes is, sometimes, severely affected by using meristic characters as tools, because of most common condition of nongenetic (phenotypical) geographic variation (Mayr, 1963). In case of anadromous and catadromous fishes the process of speciation is even more complicated. For example a freshwater form often switches over to a marine life and the hereditary divergence is started due to different environmental conditions. Changes in quantitative protein fractions are of common phenomena in the blood tissues of fishes. It is not only due to seasonal changes (Shell, 1961) but physiological change is also responsible for altering the concentrations of most protein systems. Therefore, sometimes difficulties are found in comparing blood serum protein patterns, because they are reflected in the mobility and number.

Due to the high degree of species specificity the hemoglobin patterns provide useful information in species identification as well as in phylogenetic studies. Species specificity of the hemoglobin of fishes has been reported by several methods, such as free boundary (Hashimoto and Matsuura, 1959, 1960) and paper electrophoresis (Buhler and Shanks, 1959). But the analysis of hemoglobin by starch gel electrophoresis remarkably increases the number of separated components and is useful not only in biochemical studies on multiple hemoglobins but also in the study of biochemical systematics (Tsuyuki *et al.*, 1965).

Among the many biochemical techniques available, the electrophoretic separation of proteins is very useful and the proteins of different body tissues provide useful information (De Ligny, 1969), e.g. skeletal muscle, eye lens etc. The skeletal muscle protein has been found to be very useful in classification at the generic, familial and higher taxonomic levels (Tsuyuki *et al.*, 1965; Tsuyuki and Roberts, 1966; Uthe *et al.*, 1966; Cowan, 1972) and also in most cases at the species level (Tsuyuki *et al.*, 1968). The usefulness of starch gel zone electrophoretic patterns of muscle myogen (Thompson, 1960; Tsuyuki *et al*, 1962; Tsuyuki and Roberts, 1963) in species identification, in general, is independent of the factors other than genetic. The analysis of muscle proteins from a single specimen gives reliable results, because it is highly species specific and extremely invariable within a species. The degree of similarity in muscle protein patterns has been reported

(Tsuyuki, et al., 1965) and it is very useful for phylogenetic studies.

The soluble proteins from the crystalline lenses of many vertebrates eyes have been studied by many investigators by a variety of methods. But the exact number of protein components in the lens of each species remain indefinite, which depends upon the methods used. Ranges of protein components have been reported in the bovine lens (Manski et al., 1961; Perry and Koenig, 1961; Maisel and Goodman, 1965; Spector, 1965) and in rabbit lens from 6-17 (Pope et al., 1958; Maisel and Goodman, 1965; Mason and Hines, 1966). A number of protein components have also been reported in chicken lens (Maisel and Goodman, 1965) and fish lens (Smith, 1962; Rao et al., 1964; Rabaey, 1964). Recent investigators are employing zone electrophoresis in various media to study the soluble eye lens proteins (Maisel and Goodman, 1965; Mason and Hines, 1966; Eckroat and Wright, 1969), immunochemical techniques (Manski et al., 1961; Zwaan and Ikeda, 1965) or fractionation on various ion exchange columns (Bjork, 1963, 1964; Mason and Hines, 1966). Immunochemical methods are found to be the best, but offer only qualitative results (Manski et al., 1961). Similar antigenic sites have also been reported in different lens proteins (Bjork, 1963; 1964; Rabaey, 1964). An increase of heterogeneity has also been shown in an ion exchange medium (Mason and Hines, 1966). Moreover, same or different batches of ion exchangers have also been noted due to lack of reproducibility in the fractionation of eye lens proteins and the distribution of soluble proteins were different in each of these methods. Therefore any pattern described, must mention the supporting medium, buffer, ionic strength and time of electrophoresis.

The soluble nuclear lens protein patterns of the vertebrates provide an unique molecular system for studying genetically based differences between the animals (Smith, 1962, 1965, 1966a,b, 1969b) and these protein patterns may identify separate breeding populations through proteins which occur in different frequencies or only in specific populations which is necessary for studying the effects of fishing and for managing the fishery (Smith and Goldstein, 1967; Peterson, 1969; Peterson and Smith, 1969; Smith, 1969a,b).

Electrophoretic studies on serum proteins have been carried out in marine and freshwater vertebrates (Starr and Fosberg, 1957; Engle and Woods, 1960; Nyman and Westin, 1969) and invertebrates (Woods *et al.*, 1958). Several extensive studies were made on plasma proteins by many investigators and a decrease in complexity was demonstrated from higher to lower classes of animals (Gunter *et al.*, 1961, Manwell, 1963). Albumin fraction was not reported in elasmobranches (Irisawa and Irisawa, 1954), and a very little albumin was observed in cyclostomes (Manwell, 1963). A general increase of globulin patterns complexity towards the lower forms was reported by Gunter *et al.*, (1961).

Intraspecific differences in the electrophoretic patterns of fish blood serum proteins have been reported by many investigators (Drilhon and Fin , 1963; Fine *et al.*, 1963). Recently, Nyman and Pippy (1972) observed different patterns of serum proteins of salmon from North America and Europe. Though serum protein pattern variations are common, but there are some common bands which show the species specificity.

A brief resume of different electrophoretic techniques employed in this study of proteins is given as follows;

FREE BOUNDARY ELECTROPHORESIS:

The basic principle of differential migration of ions in an electric field was practically demonstrated after the development of free electrophoresis technique of Tiselius(1937). This permits the migration of proteins without any interaction with the supporting medium and in addition the various proteins are characterized as to electrophoretic mobility. The quality of the free electrophoresis analysis depends upon the buffer system employed, the ionic strength of the buffer and the nature of the protein mixture analysed.

Free electrophoresis has been employed by many investigators (Hesselvik, 1939; Rupe *et al.*, 1958; Bjork, 1961; Perry and Koenig, 1961; Ben *et al.*, 1966; Papaconstantinou, 1967; Cobb and Koenig, 1968a,b; Cobb *et al.*, 1968a,b) to study the soluble eye lens proteins of one or more species. Except for the rabbit lens (Rupe *et al.*, 1958) where eight components were detected in free electrophoretic patterns, two or three components were found in most of these studies. Lebhart *et al.* (1947) used free electrophoresis and reported four components in the lens of blue fish. Cobb *et al.* (1968a) examined the lenses from twelve bony fishes and a cartilaginous fish by this method as well as cellulose acetate electrophoresis using tris veronal buffer at pH 8.56 and found that all the patterns were characterized by a component migrating towards the cathode. Similar conclusion was also drawn by Bon *et al.* (1964) and Maisel and Goodman (1965) in fish lens with net positive charges at pH 8.6 (tris citrate buffer). In teleost, except for the sea catfish, the electrophoretic patterns of all the species appear to be characteristic for the class. Cobb and Koenig (1968a) provided extensive resolutions of mammalian lens proteins by free electrophoresis in tris veronal buffer as well as phosphate buffer. The work of Manski and Halbert (1965) indicates that the soluble lens proteins of whale are more antigenically similar to the human lens than to the fish or lamprey lens.

The blood serum proteins were first studied over one hundred years ago but the blood plasma proteins in definitive characterization did not come until the development of more discriminatory procedures of moving boundary electrophoresis and analytical ultracentrifugation in 1930's. In fact, it was not until 1937 that Tiselius electrophoretically separated and named the α, β and γ globulin fractions.

Numerous studies have been made on plasma and serum proteins of many animals by free electrophoresis studies. Svensson (1941) observed electrophoretic results for swine serum as well as some other animals. Cooper (1946) made a similar study on artificial protein mixtures and on swine serum using barbital buffer at pH 8.6 and 7.8.

Species differences have been shown electrophoretically on serum protein fractions by Deutsch and Goodloe (1945), Deutsch and McShan (1949), Moore (1945) and observed that α, β and γ globulins are present in each animal although the relative amounts of serum components vary considerably in each of the species. Fujioka et al., (1967) analysed five rabbit sera during 19 to 28 months after birth by free electrophoresis and showed a seasonal variations in the electrophoretic patterns. Matsushita et al., (1971) reported that after 36 months of birth the electrophoretic patterns of serum protein components were not affected. Free electrophoresis technique is found to be an useful tool to study the phylogenetic relationship of animals since the plasma proteins of an animal are characteristic and distinct differences are found between patterns of closely related forms (Deutsch and McShan, 1949).

PAPER ELECTROPHORESIS STUDIES:

The free electrophoresis technique was found to be not suitable due to difficulties of handling and large number of samples can not be compared simultaneously. Therefore it did not become a popular method for the electrophoretic studies of proteins. Automatically the free electrophoresis technique was replaced by paper electrophoresis. The use of paper electrophoresis was reported first by Koenig in the the year 1937. von Klobusitzky and Konig (1939) published one paper for the separation of a yellow pigment from a snake venom, its first use in separating protein mixtures. The ease in handling and the comparison of large number of samples at a time by this procedure has resulted in its wide application. Since the electrophoretic pattern is changed by disease, it was hoped that it might have diagnostic value, for example in agammaglobulinemia and hypogammaglobulinemia of human serum protein disease, the electrophoresis provides the best basis for diagnosis. Quantitative determination of amino acids separated by paper electrophoresis was made in 1948 by Wieland and Fischer.

A number of investigators used paper electrophoresis study of protein for the identification of many species of vertebrates. Bon (1960) examined the soluble proteins from lenses of thirteen species of fishes from European waters by paper electrophoresis. Smith (1962) reported two components from lenses of bluefin tuna (<u>Thunnus saliens</u>) and kelp bass (<u>Paralabrax clathratus</u>) and one component from Pacific albacore (<u>Thunnus germo</u>) and interspecific variations were noted. Genetic differences were also detected within yellowfin tuna by means of this method (Smith, 1965).

Maertens and Peeters (1962) reported that due to extensive interactions between the lens protein and the paper, paper electrophoresis is unreliable as a means of analysis. There is a tendency of the separated bands to become broad and indistinct due to the coarse grain of the paper. In addition the irregularities of the paper increase the difficulty of determining whether a small component remains after the correction or not. Even in the specialized investigations only the rough correction could be made. Perhaps it is due to the adsorption or binding properties of the proteins to paper strips. Therefore paper electrophoresis was not used in large scale to study the proteins.

Paper electrophoretic patterns of the plasma and serum proteins of many vertebrates have been determined under uniform conditions as a part of study of the comparative biochemistry. Cremer and Tiselius (1950) and Turba and Enenkel (1950) used paper electrophoresis for serum proteins by means of dye elution techniques. Grassman and Hannig (1950) studied photoelectric scanning of dyed paper electrophoresis patterns of serum. Dessauer and Fox (1956) described the electrophoretic plasma patterns of some amphibians and reptiles and suggested that electrophoretic patterns of plasma proteins of an animal are characteristic and offer an useful technique to study the phylogenetic relationships of animals.

CELLULOSE ACETATE ELECTROPHORESIS STUDIES:

Recent investigators have taken more interest on the electrophoresis studies of proteins in cellulose acetate strips, which produce better results than the paper. This technique is advantageous in the sense that cellulose acetate strips have fewer charges than the paper, which results in negligible adsorption of proteins and the reduction of the electrophoretic flow. In addition, the use of higher voltages has decreased the time of electrophoresis. Regarding the distribution of protein components, zone electrophoresis on cellulose acetate provides additional information. The protein components can be determined by their electrophoretic mobilities used by sedimentation analysis on the basis of size and shape.

Smith (1966a,b) reported intraspecific genetic variation on the basis of the electrophoretic patterns of nuclear lens proteins. Such intraspecific differentiation was established by the study of protein patterns obtained from separate population. Seven different protein patterns were obtained in bluefin tuna (Thunnus thynnus). Out of these seven patterns five were specific to the Australian fish, one was found in the California fish and one was identical in both the regions (Smith, 1966a,b). Besides the genetic differences, he also observed the morphological differences of the two populations. Smith and Goldstein (1967) studied nuclear lens proteins by modified cellulose acetate electrophoresis method and identified the genetic differeness within the individuals of ocean white fish (Caulolatilus princeps). Intraspecific variations were also reported by Peterson and Smith (1969) in sandbar shark (Carcharhinus milberti) where all the ten different patterns were found differing both qualitatively and quantitatively, indicating a large number of heterogeneity. Recently, Smith and Clemens (1973) studied the nuclear eye lens proteins of bluefin tuna and observed that there is a single population which exhibits local genetic differentiation on the fishing grounds.

Cobb *et al.*, (1968a,b, 1969) employed free as well as cellulose acetate electrophoresis methods on the distribution of soluble protein components of fishes, amphibians, reptiles, birds and mammals. They pointed out that a single component by free electrophoresis may be separated into two or three components by cellulose acetate electrophoresis. They also interpreted a phylogenetic relationship on the basis of soluble lens proteins according to sedimentation and electrophoresis.

Serum proteins have also been studied by many investigators by employing cellulose acetate electrophoresis. Summerfelt (1966) observed the serum proteins of golden shiner and man by this technique. Aitken and Perry (1972) studied both qualitatively and quantitatively on the serum proteins of pheasants (Phasianus colchicus) and also some birds. [illegible] *et al.* (1972) employed cellulose acetate electrophoresis in a rapid large scale screening of blood proteins on a solid support.

STARCH GEL ELECTROPHORESIS STUDIES:

The molecular sieve electrophoresis method was first developed by Smithies (1955) using starch (hydrolysed) as a supporting medium. A remarkable increase of the number of protein components was detected in human serum. It has been widely adopted as a protein fractionation method due to its sieving properties, which allow the migration of both small and large protein molecules, contrary to nonsieving agar gel, which was the principle gel forming material before 1955. The method is quite advantageous because it has the capacity to compare large number of samples at a time, with its extreme sensitivity and ease of manipulation. The consistency of the electrophoretic patterns in starch gel electrophoresis is found to be more profound than the previous methods.

This method has been widely adopted and most of the work has been done on serum proteins (Tsuyuki and Roberts, 1966; Nyman, 1966a,b; 1967a,b), hemoglobins (Tsuyuki et al., 1965, 1968; Hasnain et al., 1973a) esterases (Markert and Hunter, 1959; Nyman, 1966a, Koehn and Rasmussen, 1967; Fujino and Kang, 1968; Holmes and Whitt, 1970), muscle proteins (Tsuyuki et al., 1965, 1968) and also some other tissue proteins. Unfortunately this method has been applied least to study eye lens proteins of fishes. Barrett and Williams (1967) analysed lens proteins of five scombroides by starch gel electrophoresis and found polymorphism only in bonito, Sarda chiliensis. They concluded that this polymorphism is due to the mobility difference of a single fraction related to ontogenetic factors. Tsuyuki et al., (1968) examined 19 species of Sebastodes and one Sebastolobus and interpreted the specificity at the generic levels only.

The technique of starch gel electrophoresis helps to detect the individual genetic differences among various humoral cellular proteins of animals (Whipple, 1964). Variationsin blood serum protein patterns have also been reported by several workers (Saito, 1957; Fine et al., 1963; Hyman, 1966a, 1967a, 1970; Aida et al., 1973; Hasnain et al., 1973b) by using this technique.

Microscale modification of the Smithies (1955) starch gel electrophoresis technique has also been reported which requires less time without any loss in the resolution of protein bands (Dasma, 1963; Ramsey, 1963; Tsuyuki et al., 1966). This technique has been well adopted for its speed and convenience to study analytical work. It involves the examination of large number of samples. It is also very useful for identifying constituents during fractionation and also for the identification of genetic differences of minor components of blood serum and other tissue proteins (Tsuyuki et al., 1966; Hyman, 1969).

Many important applications of the micro-starch gel electrophoresis method have been made by modern researchers of genetics and evolutionary biology.

POLYACRYLAMIDE GEL DISC ELECTROPHORESIS:

A modification of the Smithies technique utilizes polyacrylamide gels instead of starch gels for the separation of proteins (Davis and Ornstein, 1959; Raymond and Weintraub, 1959). The polyacrylamide gel is prepared more conveniently than the starch gel. The porosity of the gel can be easily adjusted over a wide range by changing the concentration of the acrylamide. Due to the absence of the charges on the gel, the electro-osmotic flow is nil during electrophoresis. The polyacrylamide gel is transparent and easy to handle. Davis and Ornstein (1962) introduced a new technique, disc electrophoresis, for the separation of proteins after the development of polyacrylamide gels which increases the number of components and the minor zones are very clear. Its advantages lie in its unparalleled resolution of protein bands, simplicity of operation and minimal cost of reagents and materials. The disadvantages are that different samples can not be compared on the same gel and the difficulties encountered in the resolved bands quantitatively.

Many researchers employed disc electrophoresis for the separation of serum proteins (Davis, 1964), hemoglobins and other tissue proteins of different vertebrates but only few works have been reported on the soluble eye lens proteins of fishes. Eckroat and Wright (1969), employing the disc electrophoresis technique of Davis (1964), found genetic polymorphism in the eye lens proteins of brook trout (Salvelinus fontinalis). Eckroat (1971) again reported that phenotypic (geographic) variations were present in varying intensities of bands as well as the presence or absence of bands. He observed that wild brook trout had much higher concentrations of proteins than the hatchery brook trout in a lens of equal size, because a wild brook trout of a certain age is smaller and has a smaller lens than a hatchery brook trout. Dey (1972) observed variations of protein patterns in the lenses of fifteen species of rodents by means of disc electrophoresis.

Electrophoretic examination of various fish plasmas and serum proteins do not define accurate characters to be used in classification. It appears to be due to extreme diversity of different fish species. However, variations of serum proteins have been reported by many keen observers, which may be dependent upon factors other than genetics, and therefore, quite unreliable for taxonomic studies. Nevertheless , some common bands have been reported which show species specific characteristics (Tsuyuki and Roberts, 1966). The plasma proteins of rainbow trout and six species of Oncorhynchus were analysed on polyacrylamide gels by Tsuyuki and Roberts (1966) who concluded that though the species specificity is maintained but phylogenetic relationship was not distinguishable on the basis of plasma protein patterns. Thurston (1967) observed the serum protein variations in rainbow trout on acrylamide gels in various biological circumstances. Perrier *et al.*, (1973) used plasma protein patterns of the rainbow trout by disc electrophoresis for classification purposes.

CORRELATION OF FREE BOUNDARY, PAPER, STARCH GEL AND ACRYLAMIDE GEL ELECTROPHORESIS:

The identification and resolution of proteins by free boundary, paper, starch gel and polyacrylamide gel have been widely employed and each method has its advantages and disadvantages. Among the various electrophoretic methods, free boundary electrophoresis is the only method which gives the accurate mobility values. Therefore it is only the reliable procedure for determining the isoelectric point and mobility curves. Though it is less sensitive than starch gel or immunoelectrophoresis, this method is best suited for quantitative study of proteins. Both free boundary as well as paper electrophoresis may be used for quantitative study but due to ease of handling, simplicity and also analysis of multiple samples simultaneously, paper electrophoresis may be preferred. The high resolution starch gel electrophoresis has been found to be well suited to systematic research for population studies though it has less quantitative value. It also provides a very high sensitive test for homogeneity. The acrylamide gel is more conveniently prepared than the starch gel and the porosity of the acrylamide gels readily adjusted over a wide range to suit the molecular size of the proteins. But large number of samples can not be compared on the same gel. Therefore micro starch gel electrophoresis of Tsuyuki *et al.* (1966) has been preferred to study the eye lens and serum proteins, where large number of samples can be compared on the same gel in a short time.

Inspite of great applicability of various electrophoretical techniques for the separation of proteins, very little attention has been paid in India to study the proteins electrophoretically. Except for the preliminary work of Chandrasekhar (1959) and Das (1961), no other information dealing with fish proteins is available. Moreover, publications under the general topics, biochemical systematics, mainly based on starch gel electrophoretic studies of various proteins have not so far been reported. On the contrary, in other countries chemotaxonomic studies subjected to fishes are extensive.

Therefore the present work was undertaken to study in detail electrophoretically, using starch gel as a supporting medium, the serum proteins and eye lens proteins of some freshwater fishes of Aligarh, which may be helpful for taxonomic purposes. The data collected over a period of three and half years is being presented here in the form of this thesis.

Chapter I deals with the preparation of serum, lens extracts, dialysis of the lens extracts, hydrolysation of starch, preparation of buffers, experimental procedures for starch gel electrophoresis and staining methods for detecting the proteins and enzymes.

Chapter II deals with the electrophoretic characteristics of eye lenses of some freshwater fishes of Aligarh.

In Chapter III the characteristics and differences in the protein patterns of whole, cortical and nuclear eye lens of _Ophicephalus punctatus_ (Bloch) and _Ophicephalus gachua_ (Ham.) have been compared.

Chapter IV is devoted to the study of the solubility of whole eye lens, cortex and nuclear lens proteins of _Ophicephalus punctatus_ (Bloch) in different concentrations of sodium chloride solutions.

The thermostability differences of the proteins of two closely related species _Ophicephalus punctatus_ (Bloch) and _Ophicephalus striatus_ (Bloch) are discussed in Chapter V.

Chapter VI deals with the blood protein studies of some freshwater fishes.

CHAPTER - I

MATERIALS AND METHODS

Some common freshwater fishes of northern India, popularly known as carps, murrels and cat-fishes were selected for the present study. 26 species of fishes belonging to 8 families were studied electrophoretically for the soluble eye lens proteins and 13 species of fishes were used for serum protein studies. Fishes were collected from different localities of Aligarh district during the period of October, 1970 to March, 1974.

PREPARATION OF SERUM:

After recording the length and weight of each specimen, the caudal peduncle was severed and blood was drained out. The blood was collected in a dry, clean, sterilized plastic tube. After keeping it one hour at room temperature, it was placed in a refrigerator at 10°C for 24 hours. Serum was obtained after centrifugation at 3000 rpm for 15 minutes and utilized immediately or stored at -10°C. The sex of each fish was also recorded.

PREPARATION OF LENS EXTRACTS:

After collecting the blood, lenses from both the left and right eyes of each fish were removed by making a slit in the cornea and were blotted quickly on absorbent paper to remove foreign materials. Lenses were then placed in a dry, clean, air tight sterilized plastic tube and utilized immediately for preparing lens extracts or stored at $-4^{\circ}C$. The lens extracts were prepared in 0.910 g. saline solution equal to approximately ten times of the wet weight of the tissues at room temperature in a glass mortar with the help of a pestle. The tube of the each extract was then sealed and shook thoroughly at one hour interval during 24 hours of extraction at $10^{\circ}C$. The lens extracts were cleared by centrifugation at 3500 rpm for 2 minutes. The cleared supernatant was immediately used for electrophoresis or stored at $-5^{\circ}C$ until used for analysis.

PREPARATION OF HYDROLYSED STARCH:

The potato starch was hydrolysed according to the method of Smithies (1955) with slight modification. 150 gm of potato starch (BDH) was suspended in double volume of acetone: HCL (100:1) at $37.5^{\circ}C$ for 100 minutes instead of 45 minutes. 75 ml of 1 M sodium acetate was added to stop the reaction. Then it was repeatedly washed thoroughly with distilled water with a Buchner funnel. The starch was suspended overnight in large volume of distilled water to remove the traces of acetate. Next morning the starch was again repeatedly washed with distilled water and also once with sodium borate buffer (gel) of Smithies (1955). Finally it was dehydrated with acetone and dried at 45°C in a thermostat.

PREPARATION OF BUFFERS:

The buffer systems employed here were prepared according to the method of Smithies (1955) for eye lens proteins. The discontinuous buffer systems described by Poulik (1957) and Ashton and Braden (1961) were employed for serum proteins, transferrins and lipoproteins. Serum esterases were run in the buffer suggested by Markert and Faulhaber (1965).

PREPARATION OF GEL:

13 gm of hydrolysed starch was added to 100 ml of gel buffer. The mixture was heated with constant and vigorous stirring by a glass rod until the starch grains ruptured. When a viscous homogenous solution was obtained, the flask was removed from the heater. The air bubbles were removed from the gel by a very short degassing step of about 1-2 secs with a negative pressure. But for the temporary stations Smithies (1955) degassing step was avoided by letting the buffer run on flask walls and by gently swirling the flask until the solution was clear and homogenous. The hot viscous starch solution was then poured into perspex moulds of 6" x 1½" x ¼", whose walls were already coated with mobil oil except the bottom where polyethylene film was placed. Only one side (broader side) of this mould was fixed temporarily with the

help of cellulose self sticking tape and the other three walls were permanently fixed. Polyethylene film was used to cover the gels, which were solidified at $5^{\circ}C$ in a refrigerator. Satisfactory gels were obtained in 2.5 hrs for electrophoresis. Freshly made gels were found superior than the gels stored ones, either in cold or at room temperature for 24 hours.

SLICING AND INTRODUCTION OF SAMPLES:

One wall, which was temporarily fixed by cellulose self sticking tape, was removed and 1 mm thick glass sheet of exact cross section of gel mould was inserted to the bottom of the mould. Automatically the gel of 1 mm thickness was pushed up uniformly above the walls and the gel was sliced uniformly by moving a thin wire guage along the edge of the mould. A thin polyethylene film of exact cross section of the gel was placed to remove air bubbles from the gel. With the help of the polyethylene film as well as to take advantage of the stickiness of the surface of the gel, the thin slice was slowly detached from the rest of the gel. The other side of the gel slice was covered similarly with thin polyethylene film. A glass sheet of same dimensions was again inserted below the previously inserted glass sheet and in this way eight gel slices of approximately equal dimensions were cut off. All the slices were covered on both sides by polyethylene film.

For the slot formation, two blades with five projections were inserted transversely in the slice at a distance of about one third from one end with the help of a flat forcep. The blades were then carefully taken out and thus five slots were formed. And in this way forty slots in the eight slices were formed and with the help of pointed droppers the samples were introduced in the slots. Care was taken when covering the slices with polyethylene film after the introduction of samples that no air bubbles remained anywhere in the gel.

ELECTROPHORESIS:

The eight cell compartments as described by Tsuyuki *et al.* (1966) were used for electrophoresis. All the eight slices were placed separately, one in each of the compartment of 9.3 cms dimensions. Each side of the gel slice was used as the bridge. The gel slices were carefully adjusted in the compartments which were filled half inch below the top with bridge buffer. The terminals of the compartments of two sides were connected with the direct current power supply (Bijoy Adco Electrophoresis Unit). Electrophoresis was carried out for two hours at a constant voltage of 100 throughout the run, which represents the voltage gradient of 5 volt/cm of the width of the gel.

STAINING METHODS:

After two hours run, the current was turned off and all the gel slices were placed in a petri dish, after removing polyethylene coverings from both sides. The gel slices were then stained in the following way:

EYE LENS PROTEINS AND SERUM PROTEINS:

The following stock solution was prepared;

150 ml of methanol, 150 ml of distilled water, 30 ml of glacial acetic acid, 70 ml of glycerin and 0.25 gm of Amido Black 10B. A small amount of stock solution was poured on the gel. After 2-3 minutes the excess dye was removed by incubating it in a solution of 5 parts of methanol, 1 part glacial acetic acid and 5 parts distilled water, and washed repeatedly in the solution mentioned above for rapid visualization. After few hours the proteins appear as distinct blue bands.

After destaining, the gels were incubated for one hour in a solution containing 7% acetic acid in 50% ethyl alcohol. In this way the gel can be stored for a longer period without being damaged, later those gels were wrapped in polyethylene sheet for photography.

LIPOPROTEIN:

0.5 g Sudan Black saturated with 100 ml of 60% alcohol at boiling point, cooled and filtered twice. The gel slice was stained overnight and rinsed three times in 50% alcohol.

NITROSO-R REAGENT (FOR DETECTING TRANSFERRIN):

The following stock solution was prepared. 100 ml distilled water, 1 g hydroxylamine hydrochloride, 2.7 g sodium acetate (3 H_2O), 1.5 ml glacial acetic acid, 0.5 g Nitroso-R salt (1 hydroxy-2-nitrosonaphtalene-3, 6-disulphinic acid). The solution was poured over the gel for 15 minutes and then washed thoroughly with several changes of the solvent, methanol; acetic acid; distilled water (5:1:5). Transferrins were shown after a few hours as faint green bands. The green band was intensified by adding 5 micrograms of ferric ammonium sulfate per ml of serum. Other proteins were usually stained yellowish brown.

ESTERASES:

α naphthol acetate was diluted to a concentration of 1 percent in equal amounts of distilled water and acetone. 4 ml of this solution and 200 mg of Fast Blue RR was added to 200 ml of sodium phosphate buffer at pH 7 (Burstone, 1962). Gels were incubated for one hour at room temperature, washed with the solvent, methanol, acetic acid and distilled water (5:1:5), photographed and discarded.

CHAPTER - II

DISTRIBUTION OF SOLUBLE PROTEIN COMPONENTS IN THE EYE LENSES OF SOME FRESHWATER FISHES OF ALIGARH

INTRODUCTION

The eye lens proteins of fishes have been found to be more suitable for electrophoretic studies, because, it is made up of a large number of protein fractions and its development is continuous throughout the life. Electrophoretic studies of soluble eye lens proteins have been made recently by many investigators to study the phylogeny, ontogeny, population differences and also for taxonomical purposes (Barrett and Williams, 1967; Cobb et al., 1968a,b; Calhoun and Koenig, 1970; Smith, 1970, 1971b & c; Smith and Clemens, 1973).

Inspite of considerable amount of work done in other countries no electrophoretic analysis of soluble eye lens proteins of fishes has been done except the publication of Siddiqui and Ali, 1974. The present study, therefore, was undertaken to compare the lens proteins of 26 fish species, belonging to 8 families to find out phylogenetic as well as interspecies relationships.

MATERIALS AND METHODS

The following fish species were collected from different localities of Aligarh district (Table 1) and their lenses were obtained for electrophoretic studies : *Barbus stigma* (Cuv. & Val.), *B. sarana* (Ham.), *Labeo rohita* (Ham.), *L. calbasu* (Ham.), *L. gonius* (Ham.), *L. bata* (Ham.), *Catla catla* (Ham.), *Cirrhina mrigala* (Ham.), *C. reba* (Ham.), *Chela bacaila* (Day), *Mystus seenghala* (Sykes), *M. cavasius* (Ham.), *M. vittatus* (Bloch), *M. tengara* (Day), *M. bleekri* (Day), *Wallagonia attu* (Bloch), *Callichrous bimaculatus* (Bloch), *Clarias batrachus* (Linn.), *Heteropneustes fossilis* (Bloch), *Notopterus chitala* (Ham.), *N. notopterus* (P), *Eutropiichthyes vacha* (Ham.), *Ophicephalus punctatus* (Bloch), *O. gachua* (Ham.), *O. striatus* (Bloch) and *O. marulius* (Ham.).

Preparation of lens extracts, dialysis process and experimental procedures for starch gel electrophoresis have been described in chapter I.

RESULTS

The physical appearance of eye lenses of all the species of fish were more or less similar. The lenses were spherical in shape with a hard fibrous nuclear core and a gelatinous cortex, although varying in size from species to species.

The soluble lens protein patterns were characterized by the presence or absence of the number of protein fractions, their mobility, staining intensity, width and resolution of the protein fractions. The migration of soluble eye lens proteins on starch gel were both on the cathodal and anodal sides. The characteristics of the protein bands of different species are described below:

Barbus stigma:

Ten fractions are detected in the eye lens electrophorogram of *B. stigma* (Table 2A, Fig. 1A). Four fractions are observed towards the cathode. One slow migrating protein component (i), closest to the point of origin on the cathodal side is lightly stained. A second, broad and well stained protein fraction (k), is next to the point of application. Besides, a pair of farthest migrating protein fractions (m & n) from the zone of sample application on the same side are darkly stained and slightly separated from each other. Towards the anode are six fractions. Three slow migrating fractions (a, b & c) are narrow, moderately stained proteins and the remaining fractions (d, e & f) are broad and darkly stained.

Barbus sarana:

Nine protein fractions are obtained in the electrophoretic pattern of *Barbus sarana* (Table 2B, Fig. 1B). Five and four bands are found on the cathodal and anodal side respectively. Towards the cathode a pair of farthest migrating protein fractions are similar to the fractions (m & n) of P. stigma. The fraction nearest to the point of origin on the same side is narrow and lightly stained, but next to this band, (j) is broad and darkly stained. Another fraction (k) is broad but lightly stained with very poor resolution. All the anodic migrating components are heavily stained except the slow migrating fraction (b), which is moderately stained.

Labeo rohita:

Nine fractions are produced in the electrophoretic pattern of L. rohita (Table 2C, Fig. 1C), five of which migrate towards the cathode. Farthest migrating three cathodal components (k, m & n) are darkly stained and separated from the remaining components, which are lightly stained and poorly resolved. The fraction nearest to the point of sample application on the anodal side is lightly stained, while the farthest migrating fractions are heavily stained (particularly the fraction d) and close with each other.

Labeo calbasu:

Electrophoretic pattern of L. calbasu consists of seven fractions (Table 2D, Fig. 1D), of which four fractions are found towards the cathode. All the fractions are intensely stained, except the farthest migrating fraction (n), which is well stained.

Three anodic migrating components are broad and heavily stained, except the fraction (e), which is moderately stained.

Laboo gonius:

The eye lens electrophorogram of *L. gonius* consists of eight fractions (Table 2E, Fig. 1E). There are four fractions toward the cathode. On the cathodal side the farthest migrating fraction ( ) is well stained, while the remaining fractions on the same side are narrow and lightly stained. Towards the anode are also four fractions, of which fraction (b), nearest to the the point of sample application is lightly stained. Next to this band, three more bands are close together, of which the fraction (d) is broad and darkly stained.

Laboo bata:

Electrophoretic patterns of proteins extracted from the lens of *L. bata* produced two different patterns (Fig. IF & F_1). Pattern F was observed from the lens proteins of the fishes of river Ganga while pattern F_1 was produced from the fish eye lens of River Kali and Panathi.

Pattern F consists of eight fractions (Table 2F), five are on the cathodal side. Farthest migrating three fractions (k, m & n) are well stained, while slow migrating two fractions (i & j) are intensely stained. Cathodic migrating three fractions are close together and broad and well stained.

Pattern F_1 (Table 2 F_1) consists of seven fractions. This pattern differs from the pattern F in that the second farthest migrating fraction (j) towards cathode is absent and the anodic migrating components are comparatively lightly stained.

Catla catla :

The protein pattern of catla catla reveals ten fractions, four of which are towards the cathode (Table 2G, Fig. 1 G). Fraction(i) is lightly stained while next to it other fractions are well stained. There are six fractions on the anodal side of which component (s), is narrow and lightly stained. Remaining fractions are narrow and moderately stained, except the fraction (o), which is broad and darkly stained.

Cirrhina mrigala:

Lens protein pattern of C. mrigala consists of nine fractions (Fig. 1H). On the anodal side six bands are observed, while three fractions are detected towards the cathode. The fractions (k, m & n) on the cathodal side are darkly stained, but the farthest migrating pair bands are close with each other. All the anodic components are uniformly narrow, and moderately stained. They are well separated from each other.

Pattern H_1 is produced from the eye lens of an immature fish of C. mrigala and reveals eight fractions (Table $2H_1$, Fig. $1H_1$). All the cathodic components are similar to pattern H

(Fig. 1), while on the anodal side one slow migrating protein fraction (a), closest to the point of origin is absent. Remaining fractions are similar to that of an adult fish.

Cirrhina reba:

Eight fractions are produced in the electrophoretic pattern of *C. reba* (Table 2I, Fig. 1I). Three fractions are towards the cathode, which are similar to the cathodic components of *C. mrigala*, except that the bands show less staining intensity. On the anodal side five fractions are found. Nearest to the point of sample application, fraction (b) is well stained. Next to this band one protein zone is very lightly stained. Farthest migrating remaining protein compoents are narrow and moderately stained.

Chola bacaila:

The Electrophoretic pattern reveals only five fractions (Table 2J, Fig. 1J), two are found on the cathodal side which are the farthest migrating bands. They are well stained and close together. Three fractions are towards the anode. Except the fraction (d), which is broad and darkly stained, remaining fractions are narrow and lightly stained.

Mystus seenghala:

Seven components are observed in the electrophorogram of *M. seenghala* (Table 3A, Fig. 2A). Of these four are towards the cathode. Two fractions (l & m) are darly stained. Remaining slow migrating compoents (i & k) are narrow, lightly stained with very poor resolution. There are three fractions on the anodal side, of which fraction (b) is very narrow and lightly stained, while other two fractions (d & e) are intensely stained.

Mystus cavasius:

Proteins extracted from the eye lens of *M. cavasius* reveals seven fractions (Table 3B, Fig. 2B). Three cathodic migrating protein components are narrow and lightly stained, while the fraction (i) is intensely stained. Three fractions are observed towards the anode. The farthest migrating fraction (e) is less intensely stained than the remaining two fractions (c & d).

Mystus bleekri:

Electrophoretic pattern of *M. bleekri* reveals seven fractions (Table 3C, Fig. 2C). Four fractions are observed on the cathodal side and three are towards the anode. All the fractions are more or less uniformly and intensely stained. One farthest migrating fraction (e) on the anodal side and a fraction (i) nearest to the point of origin on the cathodal side are very narrow and lightly stained, while fraction (l) is darkly stained.

Mystus vittatus:

Six fractions are produced in the electrophoretic pattern of *M. vittatus* (Table 3D, Fig. 2D). Towards the cathode are four fractions. Only the fraction (1) is intensely stained otherwise other fractions on the same side are poorly stained with poor resolution. Two farthest migrating fractions on the anodal side are very narrow and lightly stained.

Mystus tengara:

Five fractions are present in the eye lens electrophorogram of *Mystus tengara* (Table 3E, Fig. 2E). On the cathodal side, there are three fractions, which are moderately stained. Towards the anode are two lightly stained fractions (d & e) similar to *M. vittatus*.

Wallagonia attu

Proteins extracted from the eye lens of *W. attu* consist of six fractions (Table 4A, Fig. 3A). Three fractions are on either side of the point of sample application. The nearest fraction to the point of origin on the cathodal side, is lightly stained while other two fractions are intensely stained. On the anodal side one farthest migrating fraction (e) is lightly stained and remaining fractions are moderately stained.

Callichrous bimaculatus:

Seven fractions are produced in the electrophoretic patterns (Table 4B, Fig. 3B). Three and four fractions are found towards the anode and cathode respectively. Cathodally migrating components are clearly separated from each other. On the anodal side two fractions (c & d) are heavily stained and show a tendency to fuse with each other. Remaining fraction (a) closest to the point of origin is lightly stained.

Clarias batrachus:

Proteins extracted from the eye lens of *C. batrachus* consist of seven fractions (Table 4C, Fig. 3C). Five fractions on the cathodal side are well separated from each other, narrow and intensely stained. Two other fractions on the anodal side are well stained.

Heteropneustes fossilis:

Seven fractions are obtained in the lens electropherogram of *H. fossilis* (Table 4D, Fig. 3D). Two farthest migrating protein fractions (l & m) on the cathodal side are lightly stained and close together. One lightly stained fraction is also present near the point of origin on the same side. Four fractions are found on the anodal side. All the fractions are uniformly and moderately stained.

Eutropiichthys vacha:

Seven fractions are observed on the electropherogram (Table 4E, Fig. 3E), of which three protein zones are found on the cathodal side and four on the anodal side. All fractions on both sides are well stained and clearly separated from each other.

<u>*Notopterus chitala*</u>:

Four fractions are obtained on either side of the point of sample insertion (Table 4F, Fig. 3F). Cathodic migrating components are well stained. On the anodal side, except the farthest migrating fraction (o), which is lightly stained, all the protein zones are intensely stained.

<u>*Notopterus notopterus*</u>:

Six fractions are produced (Table 4G, Fig. 3G). Towards the cathode only two farthest migrating protein fractions are present and of which fraction(m)is quite darkly stained than the remaining one and both are close together. All the anodic components are similar to the anodic components of *N. chitala*.

<u>*Ophicephalus punctatus*</u>:

The electrophorogram of *O. punctatus* consists of twelve fractions (Table 5A, Fig. 4A). Six bands are on either side of the point of sample application. The farthest migrating protein band (o) on the cathodal side is well stained and clearly separated from the next two bands (m & n), which are darkly stained and separated by a narrow space. Other three bands (j, k & l) are narrow, lightly stained and of poor resolution.

On the anodal side there are also six fractions which are well separated with each other. The band (a) closest to the point of sample application, is very lightly stained. The next other three bands (b, c, & d) are stained well, particularly the second one (b) which is broader and longer. The farthest migrating other (e & f) bands are lightly stained, and of poor resolution.

Ophiocephalus gachua:

Eleven fractions are obtained on the electrophorogram of the eye lens proteins of O. gachua (Table 5B, Fig. 4B), of which seven fractions are on the cathodal side. All the cathodal migrating fractions (i, j, k, l, m, n, o) are well stained. However, fractions (l & m) show a tendency to fuse with each other. On the anodal side, nearest to the point of sample application, there is a very narrow and lightly stained fraction (a). The second and third fractions (b & c) are darkly stained (specially the second one which is very broad and darkly stained). The farthest migrating two bands (e & f) are absent unlike those of O. punctatus.

Ophiocephalus striatus:

Proteins extracted from the eye lens of O. striatus reveal ten fractions (Table 5C, Fig. 4C). On the cathodal side there are six fractions. To some extent all the fractions are uniformly stained. The farthest migrating protein bands (m, n & o) are well stained and clearly separated with each other. Other two bands (k & l) are quite far from the band (i) which is just near to the point of sample application. On the anodal side there are four bands, of which band (b) is darkly stained. Other bands are also intensely stained. Like the pattern of O. marulius, the farthest migrating protein band (e) is absent but unlike the other three species of Ophicephalus a narrow lightly stained band (a) is absent.

Ophicephalus marulius:

Ten fractions are found in the eye lens proteins of O. marulius (Table 5D, Fig. 4D). Five bands are on either side of the point of sample application. On the cathodal side, the farthest migrating protein bands (m & n) are well stained. Other bands are also intensely stained. On the anodal side, band (a), which is nearest to the point of origin, is intensely stained. The remaining farthest migrating protein bands (b,c,d & f) are also intensely stained and well separated with each other similar to those of O. striatus.

DISCUSSION

Electrophoretic patterns produced by the eye lens proteins of observed fishes are not only characteristic to different families, but also to different genera and even to different species of the same genus. The occurrence of multiple protein fractions with different electrophoretic mobility in fish lens electropherograms are most important for species identification as well as for phylogenetic studies. Similar observations have been made in bird lens (Rabaey, 1962) and fish lens (Rabaey, 1964; Cobb *et al.*, 1968a; Smith, 1968), which seems to be a common characteristic of the lens proteins. The multiple protein patterns are highly specific and this phenomenon is found to be most reliable biochemical criteria in the study of animal relationships. The variability of low molecular weight lens proteins among the fishes is important in the sense that it is practically species specific. The high molecular weight of proteins on the cathodal side are much more stable in the same family and even in the same order.

At species level, identity in electrophorograms of two closely related species is often found in whole eye lens proteins and this reflects the limitation for detecting genetic differences. One or more proteins with the same mobility at this level show differences in primary structure. Similar conclusion was also drawn by Tsuyuki *et al.*, 1968. Differences in the whole proteins are not detected by electrophoresis, which could result readily by substitutions in the polypeptide chain of hydrophobic by hydrophobic amino acids or of hydrophilic by hydrophilic amino acids of the same charge (Epstein, 1964, 1966).

Biochemical evidence derived from the electrophoretic studies of soluble lens proteins has been used to establish taxonomic relationships (Rabaey, 1964; Cobb *et al.* (1968b). Phylogenetic relationship among the species of the same genus, as well as correlationship with other species has been observed on the basis of information derived from the results of biochemical studies. The electrophorograms were sufficient enough to prove the electrophoretic nature of the inherent chemical differences between them.

Species specificity and interspecific variations were observed by protein pattern differences, which are based on the characteristics of the protein fractions. A remarkable similarity was found in the electrophoretic patterns of B. stigma and B. sarana. This is especially true among the farthest migrating protein fractions of the point of origin. Species specific characteristics were demonstrated by low molecular weight and slow migrating protein components. This phenomenon has also been described by Hobsey (1964) in his examination of the whale lens proteins by immunoelectrophoresis.

Polymorphism of the soluble lens proteins was not demonstrated in genus Labeo, except in L. bata, where the polymorphism was exhibited on the cathodal side of the origin. The polymorphism is a simple one and it may be due to hereditary differences. Patterns F & F_1 (Fig. 1) were obtained from river Ganga and from river Kali and Panothi canal respectively. Therefore, the uniformity of the two protein patterns, produced by fishes obtained from two different geographical places, clearly reveals the reliability of the electrophoretic method. It may be concluded from the results that electrophoresis of soluble eye lens proteins may identify separate breeding populations.

Soluble lens protein polymorphism has been reported in bonito, _Sarda chiliensis_ (Barrett and Williams, 1967) and was found to be due to ontogenetic factors. Genetic polymorphism has also been observed in the whole eye lens proteins of brook trout (Eckroat and Wright, 1969; Eckroat, 1971).

Four species of _Labeo_ were more or less similar in the eye lens electrophoretic patterns. Only few variations were found in the slow migrating protein components. Three components, which were farthest moving on both sides of the point of origin, were common in all the species, though differed in staining intensity. It indicates that they belong to same genus.

Soluble eye lens electrophoretic patterns reveal a close relationship between _L. rohita_ and _L. bata_ (pattern F, Fig. 1). Further, _L. gonius_ appears to be closely related with _L. bata_, but not with _L. calbasu_. Close phylogenetic relationship was also observed between the genus _Labeo_ and _Barbus_ but quite different patterns were found in other carps. While _C. catla_ and _C. mrigala_ were found to be closely related with each other. Phylogenetic relationship of closely related species has been reported on the basis of the distribution of soluble proteins by sedimentation and electrophoresis (Maisel and Goodman, 1965; Cobb _et al._, 1968b).

In _C. mrigala_ variations in the composition of soluble eye lens proteins of immature and mature fishes were observed.

The immature fish lacked one slow migrating component on the anodal side. It indicates that this band appears when the fish attains sexual maturity.

A common phenomenon was also noticed in the genus *Mystus*, where variations were more pronounced among the slow migrating low molecular weight proteins. Species specific characteristics are maintained in all the species of *Mystus*. A remarkable similarity was noticed on the cathodal side of the point of sample insertion. Evidence of the present study indicates a close similarity between *M. seenghala* and *M. cavasius*. On the contrary, *M. vittatus* appears to be closely related with *M. tengara*. Further, a relationship was also noticed between *M. cavasius* and *M. bleekri*. Similar relationship has been established on the basis of morphological characters (Jayaram, 1953). Among other cat fishes, species specific characteristics are also maintained. Intraspecific differences were not found in any one of the species of *Mystus*.

Electrophoretic patterns of *N. chitala* and *N. notopterus* show a close relationship with each other.

Interspecific differences among the four species of *Ophicephalus*, namely, *O. punctatus*, *O. gachua*, *O. striatus* and *O. marulius* were clearly observed in the electrophoretic patterns of eye lens proteins, phylogenetic relationships among these species were also observed. Constancy and species specificity in the eye lens proteins of these species are obtained. Four and three bands are common on the cathodal and anodal sides respectively. On the cathodal side variations are found near the point of sample application, whereas, on the anodal side variations are more pronounced in the farthest migrating protein fractions. A pair of farthest migrating protein fractions towards the cathode are characteristic features in the electrophoretic patterns of the species of Ophicephalus. Nevertheless, these two bands appear to be of different composition in different species. Smith (1970) also demonstrated interspecific differences in the electrophoretic patterns of nuclear lens proteins of yellowfin and bigeye tuna.

Phylogenetic relationship among the four species of Ophicephalus has been established on the basis of the nature of the soluble electrophoretic patterns. It appears from the eye lens protein patterns that O. punctatus is phylogenetically more related to O. gachua than the other two species, as the number of bands decrease from O. punctatus to O. marulius through O. gachua, and O. striatus. Srivastava (1968) came to the same conclusion on the basis of the study of morphological characters. Further, the electrophoretic patterns show a close relationship between O. striatus and O. marulius. Similarities were also noted between O. striatus and O. gachua, and therefore O. striatus is regarded to occupy a position in between O. gachua and O. marulius. A decrease in complexity of proteins takes place from O. punctatus to O. marulius. Thus on the basis of the electrophoretic patterns of eye lens proteins, the four species can be arranged in the following ascending evolutionary order:

1. O. marulius,
2. O. striatus,
3. O. gachua and
4. O. punctatus

The concentration of soluble eye lens proteins of the species of Ophicephalus found to be constant in different age groups, although Yanshi et al. (1966) reported progressive decrease in the concentration of eye lens proteins of vertebrates with age. Sex, size and environmental differences were also found to have no effect on proteins. Eckroat and Wright (1969) observed no variations in the eye lens protein patterns of brook trout (Salvelinus fontinalis) in relation to sex, size and age differences.

A high degree of similarity was found between the proteins of left and right eye lenses of the same individual of each species studied. This indicates that the electrophoretic analysis produce highly similar results. Identical electrophoretic patterns of right and left eye lens proteins have also been reported in yellowfin tuna (Barrett and Williams, 1967).

No observable differences were noted from the same supernatant solutions when stored in frozen condition. Similar findings were also observed by Barrett and Williams (1967).

SUMMARY

The distribution of the soluble eye lens proteins of 26 species, belonging to 8 families were studied in detail and compared with other related species. Species specific characteristics are maintained in all the species. Low molecular weight proteins among the species are found to be more variable than the farthest migrating high molecular weight proteins.

Lens protein polymorphism was observed only in L. bata. Two patterns were found from two different localities of Aligarh. Interspecies relationship was observed among the species of Labeo. Protein variations were observed between mature and immature fishes of C. mrigala.

Phylogenetic relationship was established on the basis of protein patterns among the species of genus Mystus. Relationships, based on biochemical characters support the morphologically based relationship.

On the basis of the electrophoretic analysis of soluble eye lens proteins, the four species of Ophiocephalus could be phylogenetically arranged in the following ascending evolutionary order (1) O. marulius, (2) O. striatus, (3) O. gachua and (4) O. punctatus.

No differences were found to be related to sex, size and environment. The electrophoretic patterns of right and left eye lens proteins produced similar results.

TABLE 1. NAMES, PLACES OF SAMPLING, SIZE RANGE AND NUMBER OF FISHES USED

Species	Number of individuals	Size range (cm)	Locality
Barbus stigma	150	4 - 6	Chautal pond, Aligarh
	195	4 - 5.5	Local market, Aligarh
Barbus sarana	95	6 - 10	Lake Sheikha and Panethi canal, Aligarh
	115	7 - 10	Local market, Aligarh
Labeo rohita	65	20 - 28	River Ganga, Aligarh
	50	20 - 30	Local market, Aligarh
Labeo calbasu	20	13 - 26	River Kali, Aligarh
	59	24 - 30	Local market, Aligarh
Labeo gonius	10	16 - 26	River Kali, Aligarh
	150	20 - 26	Local market, Aligarh
Labeo bata	55	15 - 20	River Ganga, Aligarh
	195	20 - 26	River Kali and Panethi canal, Aligarh
Catla catla	52	22 - 35	River Kali, Aligarh
	28	20 - 30	Local market, Aligarh
Cirrhina mrigala	62	8 - 30	River Kali, Aligarh
	50	25 - 32	Local market, Aligarh
Cirrhina reba	150	13 - 20	River Kali, Aligarh
	57	15 - 19	Local market, Aligarh
Chela bacaila	55	10 - 12	River Kali, Aligarh
Mystus seenghala	59	13 - 30	River Kali, Aligarh
	13	25 - 32	River Ganga, Aligarh

Continued...

TABLE 1 (CONTINUED)

Species	Number of individuals	Size range (cm)	Locality
Mystus cavasius	250	9 – 13	River Kali, Panethi canal and local market Aligarh
Mystus bleekri	195	10 – 14	River Kali and Panethi canal, Aligarh
Mystus vittatus	550	8 – 12	River Kali, Sheikha ditches and local market, Aligarh.
Mystus tengara	332	10 – 12	Panethi canal and local market, Aligarh
Wallagonia attu	120	30 – 42	River Kali and River Ganga
Callichrous bimaculatus	150	15 – 22	Panethi canal and local market, Aligarh
Clarias batrachus	992	20 – 30	Sheikha ditches and local market, Aligarh
Heteropneustes fossilis	135	19 – 24	Sheikha ditches and local market, Aligarh
Eutropiichthyes vacha	25	10 – 14	River Kali, Aligarh
Notopterus chitala	20	20 – 30	River Kali, Aligarh
Notopterus notopterus	168	10 – 17	River Kali, and local market, Aligarh
Ophiocephalus punctatus	244 1033	10 – 18 11 – 19	Sheikha ditches and River Kali, Aligarh Local market, Aligarh
Ophiocephalus gachua	19 50	11 – 18 10 – 17	Panethi canal, Aligarh Hasanpur, Moradabad
Ophiocephalus striatus	95 111	19 – 32 16 – 35	Sheikha ditches and Panethi canal, Aligarh Local market, Aligarh
Ophiocephalus marulius	25 19	18 – 28 30 – 38	Sheikha ditches, Aligarh Local market, Aligarh

TABLE 2. SUMMARY OF THE PROTEIN ZONES APPEARING IN THE TYPICAL MUSCLE PROTEIN ELECTROPHEROGRAMS OF BARBUS STIGMA (A), B. SARANA (B), LABEO ROHITA (C), L. CALBASU (D), L. GONIUS (E), L. BATA (F & F1), CATLA CATLA (G), CIRRHINA MRIGALA (H), C. MRIGALA (IMMATURE FISH) H1, C. REBA (I), AND CHELA BACAILA (J). VERTICAL LINE MARKS THE ORIGIN. NEITHER THE RELATIVE INTENSITIES OF THE ZONES NOR THEIR MOBILITIES ARE CONSIDERED IN THIS REPRESENTATION.

Species	Pattern	RELATIVE POSITION OF PROTEIN ZONES												
		Cathode						Anode						
		n	m	l	k	j	i	a	b	c	d	e	f	g
Barbus stigma	A	+	+	-	+	-	+	+	+	+	+	+	+	-
Barbus sarana	B	+	+	-	+	+	+	-	+	-	+	+	+	-
Labeo rohita	C	+	+	-	+	-	+	-	+	+	+	+	-	-
Labeo calbasu	D	+	+	-	+	+	-	-	-	+	+	+	-	-
Labeo gonius	E	+	+	-	+	-	+	-	+	+	+	+	-	-
Labeo bata	F	+	+	-	+	+	+	-	-	+	+	+	-	-
Labeo bata	F_1	+	+	-	+	-	+	-	-	+	+	+	-	-
Catla catla	G	+	+	-	+	-	+	-	-	+	+	+	+	+
Cirrhina mrigala	H	+	+	-	+	-	-	-	+	+	+	+	+	+
Cirrhina mrigala	H_1	+	+	-	+	-	-	-	-	+	+	+	+	+
Cirrhina reba	I	+	+	-	+	-	-	-	-	+	+	+	+	+
Chela bacaila	J	[illegible]	+	-	-	-	-	-	-	-	+	+	-	-

+ = Presence of protein zone

- = Absence of protein zone

TABLE 3. SUMMARY OF THE PROTEIN ZONES APPEARING IN THE LENS PROTEIN ELECTROPHEROGRAMS OF *MYSTUS SEENGHALA* (A), *M. CAVASIUS* (B), *M. BLEEKERI* (C), *M. VITTATUS* (D), *M. TENGARA* (E). VERTICAL LINE SHOWS THE ORIGIN. INTENSITIES AND MOBILITIES OF BANDS ARE NOT CONSIDERED HERE.

Species	Pattern	Cathode m	l	k	j	i	Anode a	b	c	d	e
Mystus seenghala	A	+	+	+	-	+	-	+	-	+	+
Mystus cavasius	B	+	+	+	-	+	-	-	+	+	+
Mystus bleekeri	C	+	+	+	-	+	-	-	+	+	+
Mystus vittatus	D	+	+	+	-	+	-	-	-	+	+
Mystus tengara	E	+	+	-	-	+	-	-	-	+	+

\+ = Presence of protein zones

\- = Absence of protein zones

TABLE 4. SUMMARY OF THE PROTEIN ZONES APPEARING IN THE EYE LENS PROTEIN ELECTROPHEROGRAMS OF *WALLAGONIA ATTU* (A), *CALLICHROUS BIMACULATUS* (B), *CLARIAS BATRACHUS* (C), *HETEROPNEUSTES FOSSILIS* (D), *EUTROPIICHTHYS VACHA* (E), *NOTOPTERUS CHITALA* (F), AND *N. NOTOPTERUS* (G). VERTICAL LINE MARKS THE POINT OF SAMPLE INSERTION. INTENSITIES AND MOBILITIES OF BANDS ARE NOT CONSIDERED HERE.

Species	Pattern	ALPHABETICAL POSITIONS OF PROTEIN ZONES										
		Cathode					Anode					
		m	l	k	j	i	a	b	c	d	e	f
Wallagonia attu	A	+	-	+	-	+	-	-	+	+	+	-
Callichrous bimaculatus	B	+	+	+	-	+	+	-	+	+	-	-
Clarias batrachus	C	+	+	+	+	+	+	-	+	-	-	-
Heteropneustes fossilis	D	+	+	-	-	+	+	-	+	+	+	-
Eutropiichthys vacha	E	+	-	+	+	-	+	-	-	+	+	+
Notopterus chitala	F	+	+	+	-	+	-	-	+	+	+	+
Notopterus notopterus	G	+	+	-	-	-	-	-	+	+	+	+

+ = presence of protein zone

- = absence of protein zone

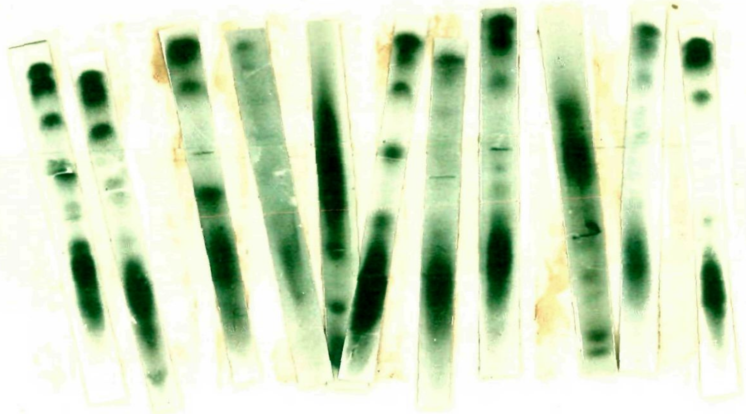
TABLE 3. SUMMARY OF THE PROTEIN ZONES APPEARED IN THE EYE LENS PROTEIN ELECTROPHEROGRAMS OF THE FOUR SPECIES OF OPHICEPHALUS. VERTICAL LINE MARKS THE ORIGIN. NEITHER THEIR RELATIVE INTENSITIES OF THE ZONES NOR THEIR ELECTROPHORETIC MOBILITIES ARE CONSIDERED IN THIS REPRESENTATION.

Species	Pattern	ALPHABETICAL POSITIONS OF PROTEIN ZONES												
		Cathode							Anode					
		o	n	m	l	k	j	i	a	b	c	d	e	f
Ophicephalus punctatus	A	+	+	+	+	+	+	-	+	+	+	+	+	+
Ophicephalus gachua	B	+	+	+	+	+	+	+	+	+	+	+	-	-
Ophicephalus striatus	C	+	+	+	+	+	-	+	-	+	+	+	-	+
Ophicephalus marulius	D	-	+	+	+	+	-	+	+	+	+	+	-	+

+ = Presence of protein zone

- = Absence of protein zone

Fig. 1. Electrophoretic patterns of soluble eye lens proteins as revealed by gel electrophoresis at pH 8.5.
A, *Barbus stigma*, B, *B. sarana*, C, *Labeo rohita*. D, *L. calbasu*, E, *L. gonius*, F and F_1, *L. bata*. G, *Catla catla*, H, *Cirrhina mrigala*, H_1, *C. mrigala* (from immature fish), I, *C. reba* and J, *Chela bacaila*.



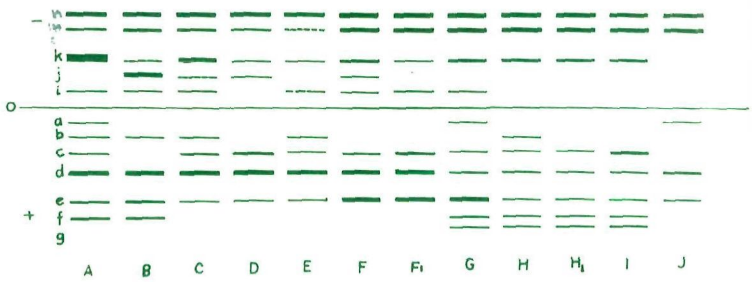
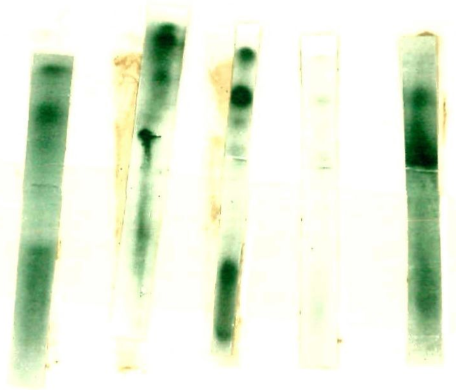


FIG. 1

Fig. 2. Starch gel electropherograms of eye lens proteins of *Mystus seenghala* (A), *M. cavasius* (B), *M. bleekri* (C), *M. vittatus* (D) and *M. tengara* (E).



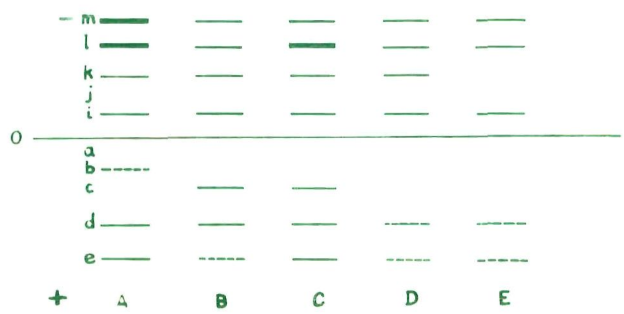
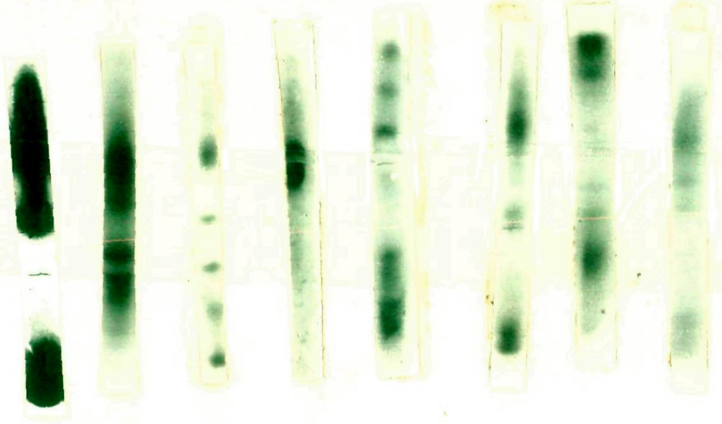


FIG 2

Fig. 3. Electrophoretic patterns of soluble eye lens proteins of A, *Wallagonia attu*, B, *Callichorous bimaculatus*, C, *Clarias batrachus* D, *Heteropneustes fossilis* E, *Eutropiichthyes vacha*, F, *Notopterus chitala* and G, *N. notopterus*.



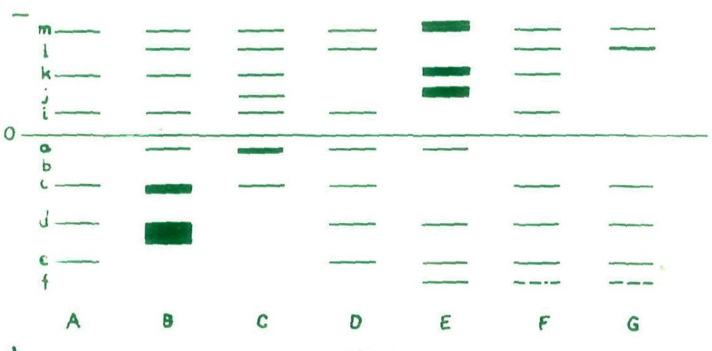


FIG. 3

Fig. 4. Starch gel electropherograms of eye lens proteins of _Ophicephalus punctatus_ (A), _O. gachua_ (B), _O. striatus_ (C) and _O. marulius_ (D).

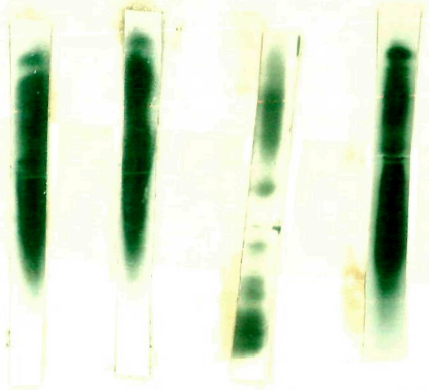


FIG. 1

CHAPTER - III

PROTEIN DIFFERENCES IN THE WHOLE EYE LENS, CORTEX AND NUCLEUS OF OPHICEPHALUS PUNCTATUS (BL.) AND OPHICEPHALUS GACHUA (HAM)

INTRODUCTION

In the course of various electrophoretic studies on the soluble eye lens proteins of vertebrates, several authors have reported the composition of soluble eye lens proteins from the cortical and nuclear regions of the lens. Papaconstantinou (1965) analysed the cortical and nuclear extracts of bovine lens by free and zone electrophoresis and compared it with the fractions obtained from DEAE cellulose chromatography but little information was given regarding the distribution of various components. Maeski et al. (1961) reported the soluble proteins from nuclear, cortical and whole lens extracts by means of agar gel electrophoresis and immunoelectrophoresis. Recently, different layers of the eye lenses of fish species have also been studied (Gutierrez, 1969; Smith, 1971a).

The purpose of the present study is to report the distribution of soluble proteins in the cortical and nuclear regions of the lens of Ophiocephalus punctatus (Bloch) and Ophiocephalus gachua (Ham) by starch gel electrophoresis and to compare these results with that observed for the whole eye lens itself. The effect of physical stress on the nature of protein bands is also reported here.

MATERIALS AND METHODS

Protein extracts from the whole eye lens, cortex and nucleus of Ophicephalus punctatus (18 cms) and Ophicephalus gachua (15 cms) were prepared in 0.018 g NaCl solution. All the extracts were dialysed at $10^{o}C$ by shaking at one hourly intervals for twenty four hours. After centrifugation at 3500 rpm for 5 minutes the supernatant solutions were used for electrophoresis.

Six specimens of Ophicephalus punctatus (15-18 cms) obtained from local market of Aligarh in living condition were kept in an aquarium and running tap water for 24 hours. Thereafter the protein extracts were prepared from the whole eye lenses, cortices and nuclei after the following individual treatments, (i) from freshly killed fish (ii) frozen once (iii) frozen, thawed, refrozen and thawed of the fish, (iv) decomposing fish in noncirculating tap water for one day and (v) for five days (vi) fish stored outdoors in the shade for two days.

The left eye lens of each fish was used for the analysis of whole eye lens proteins. Detailed description for the preparation of lens extracts has been described in Chapter I.

The cortical extracts were prepared by carefully stirring the whole eye lens in 0.018 g' NaCl solution with the help of two forceps, until all the apparent cortical material was removed. The remaining nuclei were rinsed in distilled water and the extracts were prepared in the similar way of whole eye lens extracts. The twenty four hours extractions was completed by stirring the mixture at one hour interval at 10°C.

The experimental conditions for starch gel electrophoresis have already been described in Chapter I. The buffer systems employed in this study were those described by Smithies (1955) but the pH was adjusted to 8.2 in both gel buffer and electrode buffer (gel buffer was prepared by 0.023M boric acid with 0.012M sodium hydroxide; electrode buffer was prepared by 0.3M boric acid with 0.06M sodium hydroxide adjusted to pH 8.2). The apparatus was cooled by running cold tap water.

PREPARATION OF GEL.

100 ml of gel buffer was heated to boiling and another 80 ml of the same gel buffer was admixed to 15 g of hydrolysed starch (obtained from Connaught Medical Research Laboratories, University of Toronto, Canada). The starch buffer solution was mixed with boiling buffer. It was shaken and degassed and poured into a framed plastic mould, measuring some 6" x 1½" x ½". After covering it by polyethylene film, it was placed in a

refrigerator at 10°C and after two hours it was used for electrophoresis.

RESULTS

Electrophoretic patterns of proteins produced by starch gel electrophoresis from the whole eye lens, cortex and lens nuclei of *O. punctatus* and *O. gachua* are presented in Table 6 and Fig. 5.

Whole eye lens: Twelve and eleven fractions are obtained on the electropherograms of *O. punctatus* and *O. gachua* respectively which have been described in detail in Chapter II (page 40&41).

Cortex: The protein patterns of the cortical layer of lens are more or less similar to whole eye lens proteins except the disappearance of one protein fraction. Eleven fractions are detected in the electropherogram of *O. punctatus*. On the anodal side all the components are similar to whole eye lens proteins. But on the cathodal side the slow migrating protein component (j) closest to the point of sample application has disappeared. The staining intensity of other components are similar to whole eye lens proteins.

In *O. gachua* on the cathodal side, one band (j) is lightly stained, but on the anodal side all the bands are similar to whole eye lens protein patterns.

Nucleus: The nuclear lens proteins of *O. punctatus* are purely cathodic and consist of four major fractions. The farthest migrating two protein components (n & o) are almost similar to cortex and whole eye lens proteins. Among the other fractions of nuclear lens proteins, the fraction (k) is broad and well stained but band (j) is poorly stained with poor resolution.

In the electrophoretic patterns of *O. gachua* five major bands are produced and one of them is on the anodal side similar to band (a) of whole lens and cortex. On the cathodal side the farthest migrating protein bands (n & o) are intensely stained but other two bands (j & k) are very poorly stained.

Electrophoretic separation of protein patterns from the whole, cortical and nuclear lenses of *O. punctatus* exposed to different physical conditions have been represented in Table 7 and Fig. 6.

The number of protein fractions decreased in the extracts of whole lens and cortex with the degree of physical stress (Table 7). The nuclear proteins were not more affected. The protein fractions of the anodal side were more affected indicating that some fractions of this side get quickly denaturated.

DISCUSSION

The protein patterns from the whole eye lens and cortex were more or less similar but distinctly different patterns were obtained for nucleus. In *O. punctatus* the protein components of whole eye lens resembled with that of cortex with one exception, i.e., the fraction (j) on the cathodal side closest to the sample application was not detected in cortex. This is due to the fact that the cortex comprised approximately 80-90% of the total soluble protein patterns of whole eye lens. Cobb and Koenig (1968c) concluded that 89-92 of cortical soluble protein patterns of bovine lens were similar to whole eye lens protein patterns.

Protein fractions from the whole eye lens, cortex and nucleus were aligned on the basis of similar mobilities. But it may not be the same proteins of similar mobilities obtained from the nucleus and cortex. Similar observations have also been made by Cobb and Koenig (1968c).

In the cortex of *O. gachua*, one protein fraction (j) on the cathodal side was less stained as compared to whole eye lens proteins. Among the four species of *Ophiocephalus*, one anodic migrating protein fraction (a) has been observed only in *O. gachua* in nuclear lens protein pattern, appears to be serum albumin. Smith and Clemens (1973) also reported similar protein fractions in bluefin tuna and concluded that this protein should be serum albumin, since this molecule has a highly electro-negative charge.

In the patterns of nuclear and cortical layer of lens proteins from both the species of O. punctatus and O. gachua, it has been observed that the disappearance of band is in the short migrating, low molecular weight proteins but not in the far migrating high molecular weight proteins. It means that the short migrating low molecular weight proteins are variable than the high molecular weight far migrating proteins.

Interspecific differences between the two species are clearly demonstrated in the patterns of nuclear lens proteins. There is one slow migrating protein band (a) on the anodal side in O. gachua but no such cathodic migrating protein fraction was found in O. punctatus. On the cathodal side three fractions (o, n & k) are common but k is broad and well stained in O. punctatus. Another point of difference is the absence of fraction (i) in O. punctatus. Besides more differences in the patterns of nuclear and cortical proteins are found (Table 6) than in the whole eye lens proteins. Therefore for species identification the former two could be used.

Electrophoretic separation of protein patterns from the whole eye lenses and cortices of O. punctatus exposed to different physical conditions produced variable patterns. Five patterns were produced due to denaturation of proteins. The denaturation of protein rapidly occurs in the whole eye lens and cortex and produced nongenetic protein variations (Sibley and Brush, 1967). The cortical eye lens is a metabolizing tissue, which synthesizes ribose nucleic acid and protein tripeptides (Davson, 1963) and at least one amino acid. Besides, cortical layer may contain the proteins of the aqueous and vitreous humors. Therefore, the cortex is not suitable for genetic studies, which may be easily turned over and altered by the environment. The evidence of the present study supports the above fact. The cortical proteins of _D. punctatus_ have been denatured rapidly in different environmental conditions. Changes of protein patterns were not observed in frozen fish eye lenses and it may be suitable for genetic studies.

In contrast with the whole lens and cortex, the nuclear lens proteins have been found to be more suitable for genetic studies (Smith, 1966a,b; Smith and Goldstein, 1967) because it resists denaturation. Besides, it is an inert structure, not contaminated by protein of other tissues, such as blood, nerve or muscle and also due to its constancy in quality or quantity (Langman, 1961; Halbert and Manski, 1963). These proteins are highly concentrated (Halbert and Manski, 1963), stable and readily soluble in many media e.g. distilled water, physiological saline etc. and can tolerate temperatures as high as 79°C, before precipitating (Smith, 1965) and without denaturing (Smith, 1966a).

No differences were found in electrophoretic patterns of fresh, frozen once and frozen, thawed and refrozen fish lens nuclei. Similar results were also obtained when the fish head was stored in noncirculating tap water for one day. Similar conclusion was also drawn by Smith (1971a). Some differences were observed when the fish decomposed in water for five days and when kept outdoors in the shade for two days.

Therefore, the results obtained in the electrophoretic patterns indicate that the denaturation of nuclear lens proteins was least and produced more simple electrophoretic pattern than the whole eye lens and cortical lens proteins.

SUMMARY

The soluble proteins from the whole eye lens, cortex and nucleous of two closely related species of O. *punctatus* and O. *gachua* were analysed by electrophoresis, using starch gel as supporting medium. The electrophoretic separation of protein patterns of whole lens and cortex were more or less similar but distinctly different patterns were found in nucleus. For the species identification of these two species, nuclear lens proteins were found to be more reliable source than the whole eye lens and cortex. The denaturation of protein was less in nucleus than in the cortex and whole lens.

TABLE 6. ALPHABETICAL PROTEIN ZONES IN THE WHOLE, CORTEX AND NUCLEAR EYE LENSES OF *OPHIOCEPHALUS PUNCTATUS* BL. AND *OPHIOCEPHALUS GACHUA* BL. RELATIVE INTENSITIES AND MOBILITIES OF PROTEIN ZONES ARE NOT CONSIDERED.

	ALPHABETICAL POSITION OF PROTEIN ZONES												
	Cathode							Anode					
	o	n	m	l	k	j	i	a	b	c	d	e	f
O. punctatus													
Whole eye lens	+	+	+	+	+	+	-	+	+	+	+	+	+
Cortex	+	+	+	+	+	-	-	+	+	+	+	+	+
Nucleus	+	+	-	-	+	+	-	-	-	-	-	-	-
O. gachua													
Whole eye lens	+	+	+	+	+	+	+	+	+	+	+	-	-
Cortex	+	+	+	+	+	+	+	+	+	+	+	-	-
Nucleus	+	+	-	-	+	-	+	+	-	-	-	-	-

Vertical line marks the point of origin

+ = Presence of protein zone

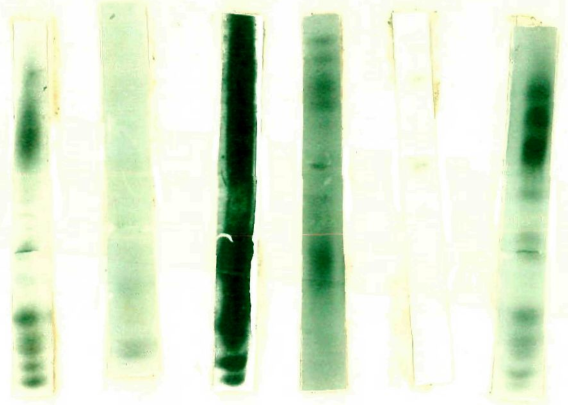
- = Absence of protein zone

TABLE 7. SUMMARY OF THE PROTEIN ZONES APPEARING IN WHOLE LENS, CORTEX AND NUCLEAR ELECTROPHEROGRAMS OF OPHICEPHALUS PUNCTATUS (BL.) UNDER DIFFERENT PHYSICAL CONDITIONS (A) FRESH AND FROZEN ONCE (B) FROZEN, THAWED AND REFROZEN (C) LEFT IN A DECOMPSOIGN FISH HEAD IN NONCIRCULATING TAP WATER AT ROOM TEMPERATURE FOR ONE DAY AND (D) FOR FIVE DAYS AND (E) STORED OUTDOOR IN THE SHADE FOR TWO DAYS. RELATIVE INTENSITIES AND MOBILITIES OF BANDS ARE NOT CONSIDERED IN THIS REPRESENTATION.

Patterns			ALPHABETICAL POSITIONS OF PROTEIN ZONES												
			Cathode							Anode					
			o	n	m	l	k	j	i	a	b	c	d	e	f
Fresh and frozen once	A	W	+	+	+	+	+	+	-	+	+	+	+	+	+
		C	+	+	+	+	+	-	-	+	+	+	+	+	+
		N	+	+	-	-	+	+	-	-	-	-	-	-	-
Frozen, thawed and refrozen	B	W	+	+	+	+	+	+	-	-	+	+	+	+	-
		C	+	+	+	+	+	-	-	-	+	+	+	+	-
		N	+	+	-	-	+	+	-	-	-	-	-	-	-
One day decomposition	C	W	+	+	+	+	+	+	-	-	+	+	-	-	-
		C	+	+	+	+	+	-	-	-	+	+	-	-	-
		N	+	+	-	-	+	+	-	-	-	-	-	-	-
Five days decomposition	D	W	+	+	+	-	-	+	-	-	+	+	-	-	-
		C	+	+	+	-	-	-	-	-	+	+	-	-	-
		N	+	+	-	-	-	-	-	-	-	-	-	-	-
Stored outdoor for two days	E	W	+	+	-	-	+	-	-	-	+	+	+	-	-
		C	+	+	-	-	+	-	-	-	+	+	+	-	-
		N	+	+	-	-	-	-	-	-	-	-	-	-	-

+ = Presence of band; - = Absence of band; W = whole eye lens ; C = cortex; N = nuclear lens.

Fig. 5. Electrophoretic patterns of protein from the whole eye lens (A), cortex (B) and nucleus (C) of *Ophicephalus punctatus* and whole eye lens (D), cortex (E) and nucleus (F) of *Ophicephalus gachua*.



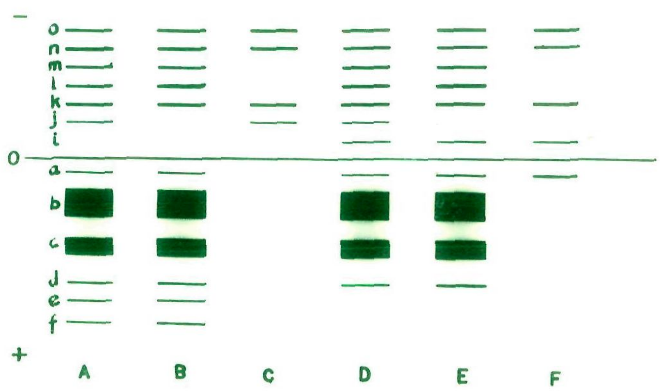
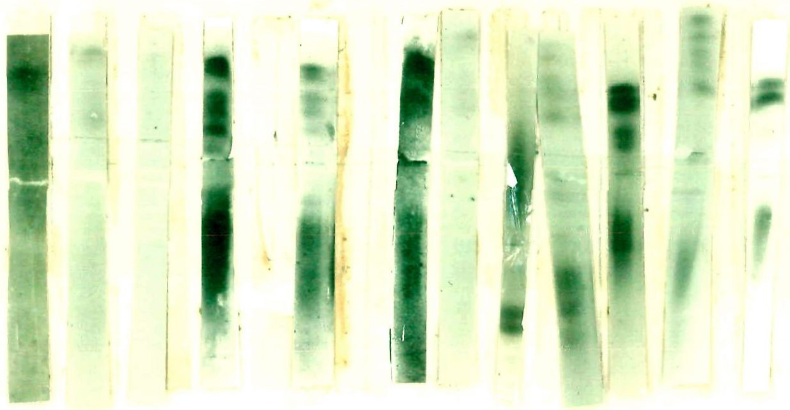


FIG. 5

Fig. 6. Electrophoretic patterns of protein from the whole (W), cortex (C), and nucleus (N) of *Ophicephalus punctatus* (A) fresh and frozen once, (B) frozen, thawed and refrozen (C) left in a decomposing fish in noncirculating tap water at room temperature for one day, and (D) for five days and (E) stored outdoors in the shade for two days.



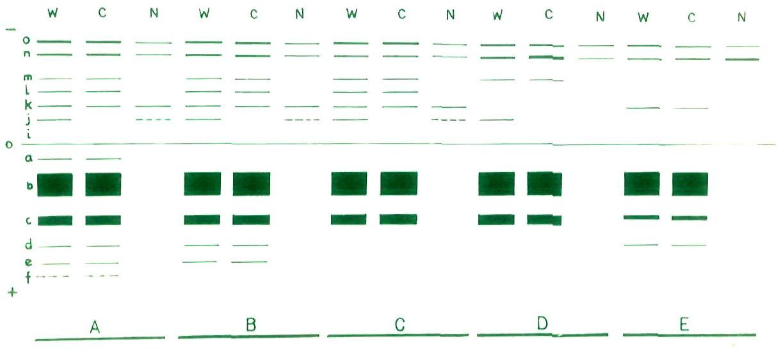


FIG. 6

CHAPTER - IV

ELECTROPHORETIC CHARACTERISTICS OF WHOLE EYE LENS, CORTEX AND NUCLEAR LENS PROTEINS SOLUBILIZED IN DIFFERENT CONCENTRATIONS OF SODIUM CHLORIDE SOLUTION OF OPHICEPHALUS PUNCTATUS (BLOCH).

INTRODUCTION

The solubility of fish eye lens proteins depends upon the extracting solution. Distilled water and physiological saline have generally been used for extracting the eye lens proteins but none of them can solubilize all classes of proteins (Sober *et al.*, 1965). Pseudoglobulins are soluble in the above two media but euglobulins are insoluble in distilled water, while albumins are soluble only in distilled water. Smith (1968) found 0.010 g/ saline solution to solubilize all classes of nuclear proteins.

Most of these electrophoretic studies have been made in cellulose acetate medium (Smith, 1969a,b, 1971a,b & c; Peterson and Smith, 1969) or by free electrophoresis (Cobb *et al.*, 1968a,b) except for the studies of Barrett and Williams (1967) and Tsuyuki *et al.*, (1966) who used the starch gel medium.

The present investigations were made to study the electrophoretic characteristics of whole eye lens, cortex and nuclear lens proteins of *Ophiocephalus punctatus*, extracted in different concentration of salt solution to find out the most suitable concentration which can solubilise all kinds of proteins. Starch gel has been used as a supporting medium.

MATERIALS AND METHODS

The fishes were collected in the size range of 15-18 cm from the local fish market. Both the left and right eye lenses of each fish were removed after making a slit in the cornea. The left lens was used for preparing whole lens extracts whereas right lens was used for cortical and nuclear lens extracts.

Whole eye lens, cortex and nuclear lens extracts were prepared separately in distilled water and in the following concentrations of sodium chloride solution (g%) ; 0.005, 0.010, 0.015, 0.018, 0.020, 0.030, 0.040, 0.060, 0.080, 0.10, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.20, 0.21, 0.22, 0.24, 0.26, 0.28, 0.42, 0.425, 0.70 and 0.85, equal to seven times of the wet weight of the tissue in a glass mortar with the help of a pestle. Dialysis and the experimental conditions for starch gel electrophoresis have been described in chapter I.

RESULTS

Whole eye lens proteins;

Seven major types of electrophoretic patterns were produced (Table 8 and Fig. 7). Proteins extracted in distilled water produced pattern A (Fig. 7A), which consists of twelve fractions. Towards the cathode are six bands. The farthest migrating band (n) is intensely stained and well separated than the rest of the components. Next to this band, other two bands (l & m) are close to each other. The remaining bands are lightly stained. On the anodal side there are also six components, of which the nearest band is very narrow and lightly stained. Other two bands (b & c) are darkly stained but the rest of the bands are poorly stained with poor resolution.

Pattern B (Fig. 7B) is produced by proteins extracted in 0.008 g saline solution. This pattern more or less resembles to pattern A and differs only in the staining intensity.

Pattern C (Fig. 7C) is produced in 0.018 g saline solution. Similar pattern was also obtained in 0.008 g saline solution. Though the number of the bands are the same as those of A and B patterns, it differs in staining intensity as well as in the separation of the bands. On the cathodal side the farthest migrating protein band (n) is well stained and well separated from the next two bands (l & m), which are darkly stained and separated by a narrow space. Other bands on the cathodal side are intensely stained. On the anodal side two bands (b & c) are broad and well stained, while the remaining farthest migrating protein fractions are narrow but well stained.

Pattern D (Fig. 7D) is of proteins extracted in salt solution of 0.030 g%. On the anodal side only five bands are present.

Other bands are identical with pattern C.

Pattern E (Fig. 7E) consists of only ten fractions. Five bands are on each side of the point of sample application. The farthest migrating protein bands on the anodal side are slightly thinner and lightly stained. On the cathodal side, all the five bands are intensely stained and separated with each other by narrow spaces.

Only nine protein fractions are obtained in 0.425 g saline solution (Pattern F, Fig. 7). Five bands on the cathodal side are similar to pattern E, but on the anodal side there are only four bands. The farthest migrating bands are very lightly stained.

Pattern G (Fig. 7G) is produced by proteins extracted in 0.85 g saline solution. It consists of eight fractions, four on either side of the point of sample application. On the cathodal side, the farthest migrating two protein fractions are well stained close to each other and separated by a wide space from the rest of the protein components, whereas on the anodal side all the bands are narrow and lightly stained with poor resolution.

Cortical lens proteins:

Six types of patterns were produced from the cortical layer of lens proteins (Table 9, Fig. 8). Pattern A is produced from the proteins extracted in distilled water and reveals nine fractions

(Fig. 8A), five on the cathodal side, which are intensely stained as pattern A of whole eye lens proteins except band j which is lightly stained. On the anodal side there are only six fractions. The farthest migrating two protein bands and one slow migrating protein fraction (a) are very lightly stained whereas band b is broad and well stained. Remaining fractions are intensely stained. Similar pattern was also obtained from the cortical lens proteins extracted in 0.005 and 0.01 g% saline solution.

Pattern B (Fig. 8B) is of proteins extracted in 0.015 and 0.020 g% saline solution. Eleven protein zones are obtained in the electropherograms. On the anodal side two farthest migrating protein fractions (e & f) are intensely stained unlike pattern A. All the fractions are well stained and well separated from each other.

Pattern C consists of nine fractions (Fig. 8C) which is produced in a series of sodium chloride solutions of 0.030 through 0.16 g%. No differences of protein fractions with pattern A are found on the cathodal side of the point of sample application but only four fractions are present on the anodal side. The farthest migrating two protein fractions (e & f) have disappeared. Other protein components are intensely stained, similar to pattern A. Only slight variations are found in the protein patterns extracted in solutions of 0.030 to 0.16 g%. Greater differences are observed when stronger than 0.19 g% saline solution is used.

Pattern D (Fig. 8D) is produced in the saline extracts of 0.28 g and reveals eight fractions. On the cathodal side all the fractions are identical with pattern C except the fraction (j) which is very lightly stained. The slow migrating fraction (e) on the anodal side of the point of sample application is absent and other fractions are lightly stained.

Pattern E (Fig. 8E) is of protein extracted in 0.42 g saline solution. Four fractions are present towardsthe cathode. One slow migrating component (j) is absent, while other bands are intensely stained. Towards the anode, there are three fractions. The intensity of staining of the protein components are similar to pattern D.

Pattern F (Fig. 8F) is of protein produced in 0.70 and 0.85 g saline solutions. On the cathodal side only three fractions (k, m & n) represent and are intensely stained. Three fractions on the anodal side are lightly stained with poor resolution.

Nuclear lens proteins:

Five patterns (Table 10, Fig. 9) were produced from the nuclear lens proteins of _O. punctatus_ related to the salt concentrations of the extracting fluid.

Pattern A (Fig. 9A) is produced when lens was extracted in distilled water. Four components are found on the cathodal side of the electropherogram. The farthest migrating bands (m & n) are intensely stained, while band j is broad and darkly stained. The remaining fraction (i) is very lightly stained with very poor resolution.

Pattern B (Fig. 9B) is produced in 0.015 and 0.010 g% saline solution. Cathodally, four migrating protein components are clearly separated and well stained. The slow migrating component (i) is intensely stained in this saline extract whereas it was very lightly stained in distilled water extracts (Pattern A). The mobility of all the fractions are same as noted for pattern A.

Pattern C (Fig. 9C) is of proteins extracted in 0.16 g% saline solution and consists of three fractions which are intensely stained. Slow migrating protein band closest to the point of sample application has disappeared.

Pattern D (Fig. 9D) is produced by proteins extracted in 0.42 g% saline solution and reveals three fractions. This pattern differs from pattern C in the respect that band j is poorly stained with very poor resolution.

Pattern E (Fig. 9E) is produced in 0.85 g% saline solution. This pattern is more or less similar to pattern D, except for weak staining intensity of the farthest migrating bands (m & n).

DISCUSSION

Variations of protein patterns in the electropherograms of whole, cortex and nuclear lens of *O. punctatus* have been clearly observed by starch gel electrophoresis and have been described in detail in chapter III. Four fractions of nuclear lens proteins having the same mobility as those of whole and cortical lens protein patterns were found but the bands were lightly stained in comparison with the whole and cortical proteins. All the nuclear protein fractions are on the cathodal side and are least affected by different concentrations of saline solutions as compared to whole and cortical proteins. Cortex being the metabolising tissue is affected most whereas the nuclear lens being an inert structure does not have any effect of nerves and blood vessels on its protein structure. Therefore solubility of nuclear proteins differ from the cortical proteins due to change in the concentration of the extracts.

Only few differences were noticed between the whole and cortical patterns in different sodium chloride concentrations. Seven different electrophoretic patterns were obtained in whole eye lens proteins of *O. punctatus* whereas six patterns in cortex and five in nucleus were observed, using the same concentrations of saline solutions for preparing the extracts. No differences were found in the right and left eye lens patterns of the same individual. Intraspecific differences have also not been reported (Siddiqui and Ali, 1974).

A great similarity was observed in whole eye lens extracts of distilled water and in 0.005 through 0.020 g saline solution, except for the width and staining intensity of the protein fractions. In cortex, one slow migrating protein component (j) closest to the point of sample application on the anodal side did not appear clearly until 0.015 g saline solution is used. In whole eye lens, fraction (i) is also intensely stained in 0.015 g saline solution. As distilled water can solubilize only albumins and pseudoglobulins, but not euglobulins, the intensely stained protein band and additional proteins appearing in this range (0.015 to 0.020 g) of salt concentrations might be euglobulins, which requires the minimum salt concentrations of 0.015 g for their solubilization. It also becomes apparent that low salt concentration (0.015 to 0.020 g) is essential and most effective for the solubilization of all classes of proteins.

Only slight differences are found in the electrophoretic patterns of whole eye lens proteins and cortical lens proteins extracted in 0.030 to 0.16 g and 0.030 to 0.222 g saline solutions. The number of protein bands are reduced in higher concentrations and the two farthest migrating protein bands on the cathodal side get broadened with a narrow space in between them. In this range of salt concentration on the anodal side only one fraction has disappeared in whole lens, whereas two fractions were absent in cortical lens protein patterns. On the cathodal side in nuclear lens proteins one slow migrating protein band (i) is absent. The disappearing protein appears to be albumin which is insoluble in this range of salt concentrations.

The number and amount of proteins further reduced in 0.425 and 0.85 g% extracting saline solutions. The farthest migrating bands on the cathodal side are broad and show a tendency to fuse with each other. It is due to decrease in the mobility of protein fractions.

The protein lost in saline solution of 0.030 g% concentration is albumin and the farthest migrating protein fraction is euglobulin extracted in 0.018 g% saline solution. The remaining farthest migrating fractions should be pseudoglobulin. This latter protein should not be euglobulin because any additional protein did not appear or solubilize in this range of salt concentrations. Similar conclusion was also drawn by Smith (1960).

Major differences have been observed in the electrophoretic patterns of whole, cortex and nuclear lens proteins extracted in distilled water and different concentrations of sodium chloride solution. The present study indicates that maximum number of proteins are soluble in very low concentrations of salt, even physiological saline (0.85 g%) gives very simple result.

The present investigation, therefore, indicates that some of the proteins are insoluble in distilled water, as well as in the higher concentrations of saline solutions. Sodium chloride solution of higher concentration as well as the physiological saline give less complex result due to decrease in the resolution of protein fractions and also due to loss of some proteins. Saline solutions of very low strength, particularly 0.015 to 0.020 g% saline solutions are more efficient and solubilize maximum number of proteins and give no salt effects on the electropherograms of whole, cortex and nuclear lens proteins of *Ophicephalus punctatus*.

SUMMARY

Whole, cortical and nuclear lens proteins of *Ophicephalus punctatus* were extracted in distilled water and in 0.005 through 0.85 g% saline solutions. Proteins were separated by electrophoresis using starch gel as a supporting medium. High concentrations of salt solutions produced salt effects on the eye lens electropherograms. Low strength salt solutions solubilized maximum number of proteins. 0.015 to 0.019 g% salt solutions were found to be most suitable to solubilize both albumins and globulins of all layers (cortex and nucleus) of lens proteins.

TABLE 8. SUMMARY OF THE PROTEIN ZONES IN THE EYE LENS ELECTROPHEROGRAMS OF *D. PUNCTATUS*, PRODUCED BY PROTEINS EXTRACTED IN DISTILLED WATER (A) AND IN SALINE SOLUTIONS OF 0.008 (B), 0.018 (C), 0.030 (D), 0.170 (E), 0.425 (F) and 0.850 (G) g% STRENGTHS. VERTICAL LINE INDICATES THE POINT OF ORIGIN. RELATIVE INTENSITIES AND ELECTROPHORETIC MOBILITIES OF PROTEIN FRACTIONS ARE NOT SHOWN IN THIS REPRESENTATION.

Patterns	Alphabetical positions of protein zones: Cathode						Anode					
	n	m	l	k	j	i	a	b	c	d	e	f
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	-	+	+	+	+	+	+	+	+
E	+	+	+	-	+	+	+	+	+	+	+	-
F	+	+	+	-	+	+	+	+	+	+	-	-
G	+	+	+	-	-	+	+	+	+	+	-	-

+ = Presence of protein zone

- = Absence of protein zone

TABLE 9. SUMMARY OF THE PROTEIN ZONES IN THE CORTEX ELECTROPHEROGRAMS OF O. PUNCTATUS. PRODUCED BY PROTEINS EXTRACTED IN DISTILLED WATER (A) AND IN SALINE SOLUTIONS OF 0.018 (B), 0.030 (C), 0.20 (D), 0.42 (E) and PHYSIOLOGICAL SALINE (0.85) (F) % STRENGTH. VERTICAL LINE INDICATES THE POINT OF ORIGIN. INTENSITIES AND MOBILITIES OF BANDS ARE NOT CONSIDERED HERE.

Patterns	ALPHABETICAL POSITION OF PROTEIN ZONES											
	Cathode						Anode					
	n	m	l	k	j	i	a	b	c	d	e	f
A	+	+	+	+	+	-	+	+	+	+	+	+
B	+	+	+	+	+	-	+	+	+	+	+	+
C	+	+	+	+	+	-	+	+	+	+	-	-
D	+	+	+	+	+	-	-	+	+	+	-	-
E	+	+	+	+	-	-	-	+	+	+	-	-
F	+	+	-	+	-	-	-	+	+	+	-	-

+ = Presence of protein zone

- = Absent of protein zone

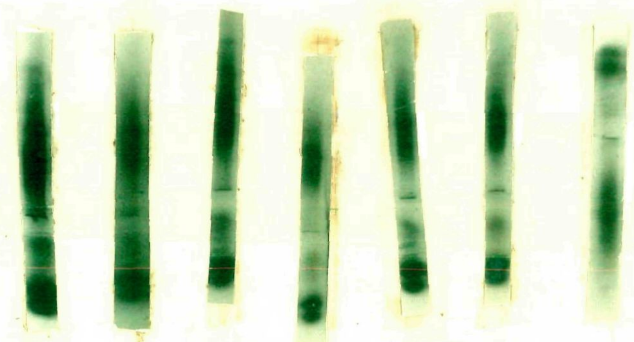
TABLE 10. SUMMARY OF THE PROTEIN ZONES IN THE NUCLEAR LENS ELECTROPHEROGRAMS OF O. PUNCTATUS, PRODUCED BY PROTEINS EXTRACTED IN DISTILLED WATER (A), 0.018 g% SALINE SOLUTION (B), 0.16 g% SALINE SOLUTION (C), 0.42 g% SALINE SOLUTION (D) AND PHYSIOLOGICAL SALINE (0.85 g%) SOLUTION (E). THE VERTICAL LINE MARKS THE ORIGIN. RELATIVE MOBILITIES AND INTENSITIES OF BANDS ARE NOT SHOWN HERE.

Patterns	ALPHABETICAL POSITION OF PROTEIN ZONES											
	Cathode						Anode					
	n	m	l	k	j	i	a	b	c	d	e	f
A	+	+	-	-	+	+	-	-	-	-	-	-
B	+	+	-	-	+	+	-	-	-	-	-	-
C	+	+	-	-	+	-	-	-	-	-	-	-
D	+	+	-	-	+	-	-	-	-	-	-	-
E	+	+	-	-	+	-	-	-	-	-	-	-

+ = Presence of protein zone

- = Absence of protein zone

Fig. 7. Starch gel electropherograms of eye lens proteins of *Ophicephalus punctatus*. The electrophoretic patterns were produced by proteins extracted in distilled water (A), and in sodium chloride solution of [illegible] (B), [illegible] (C), [illegible] (D), [illegible] (E), 0.425 (F) and [illegible] (G) μ strength.



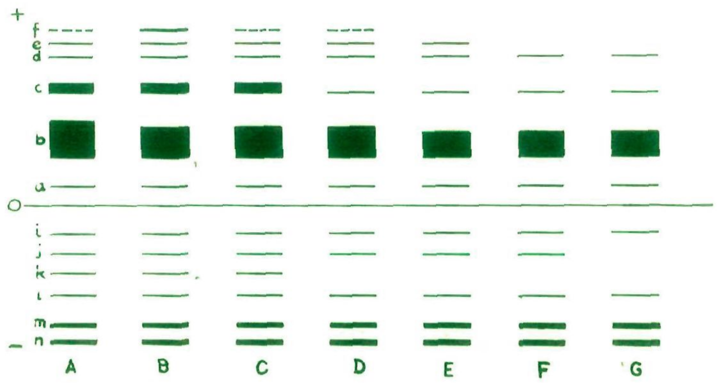
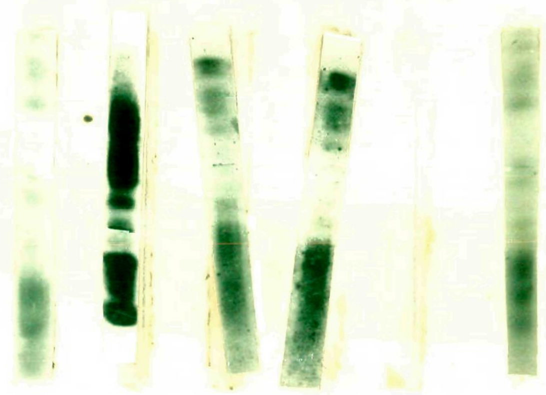


FIG. 7

Fig. 8. Electrophoretic patterns of protein from the cortex of _Ophicephalus punctatus_. The solutions used for extracting the proteins in patterns A through F are as follows: A, distilled water; B, 0.018 g% saline solution, C, 0.030 g% saline solution, D, 0.28 g% saline solution; E, 0.42 g% saline solution F, 0.85 g% saline solution.



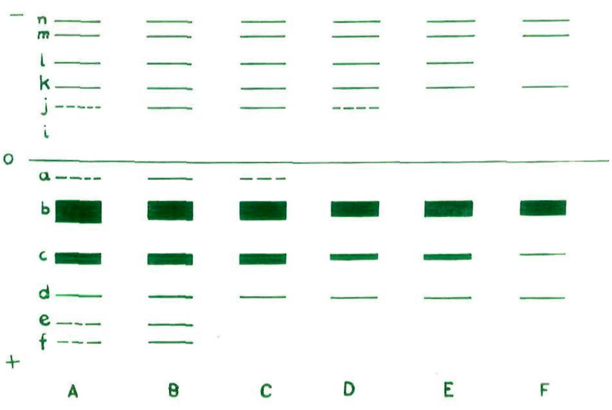


FIG. 8

Fig. . Nuclear eye lens protein patterns of *Ophicephalus punctatus*. A is produced by protein extracted in distilled water, B, 0.018 g% saline solution, C, 0.16 g% saline solution, D, 0.42 g% saline solution and E physiological (0.85 g%) saline solution. The vertical line indicates the origin.

FIG 9

CHAPTER - V

STUDIES ON THE THERMOSTABILITY OF EYE LENS PROTEINS OF TWO CLOSELY RELATED FISH SPECIES AS REVEALED BY STARCH GEL ELECTROPHORESIS

INTRODUCTION

Most of the proteins are extremely sensitive to heat. Some proteins are denatured even at room temperature. Protein denaturation may be avoided at low temperature, which is essential for the isolation and purification of proteins, and in this connection refrigerator, refrigerated centrifuge and cold rooms play an important part for the preparation and separation of proteins.

Some proteins are denatured rapidly, but some resist denaturation upto certain limit of temperature. Protein denaturation also occurs in different species at different temperatures.

The electrophoretic separation of eye lens proteins has been used by many workers for demonstrating genetically based molecular differences among the animals of different taxonomical categories and also, for the identification of separate breeding populations (Zan _et al._, 1964; Maisel and Goodman, 1965; Bennett and Williams, 1967; Cobb _et al._, 1968a,b; and Tsuyuki _et al._, 1968). Protein variations have also been observed among and within the species of some fishes from nuclear lens (Peterson and Smith, 1969; Smith, 1971b,c; Smith and Clemens, 1973).

The present study reports the effects of temperature on the eye lens proteins of two very closely related species, Ophicephalus punctatus and Ophicephalus striatus. The extracts of eye lens proteins of these two species were electrophoretically processed after the extracts were heated for different time intervals. The purpose of this study is to determine the thermostability differences of eye lens proteins of the above two closely related species of Ophicephalus.

MATERIALS AND METHODS

One lens was removed from each individual of O. punctatus and O. striatus, which were collected from local fish market of Aligarh. The lenses were stored, frozen in dry sealed containers. Other lens was removed for analysing the nuclear lens proteins. 0.015 g. saline solution was added for preparing the extracts as described in Chapter I.

Dialysis was conducted overnight at 10°C by disturbing the extracts at one hour interval. Since salt concentration affects the thermostability of proteins (Vesell and Yielding, 1966), the dialysis of lens protein extract was performed in the same salt concentration and eventually the protein concentrations of the extracts were similar. Therefore, no adjustment was necessary to equivalence the protein concentration of the extracts in order to exclude concentration differences for studying the thermostability differences.

The extracts of each species were divided into eight equal aliquots in separate test tubes. One tube of each species was placed in an ice bath and similar other tube pairs were placed into boiling water for the following time periods (sec) 1,3,5,8, 14, 18 and 24. Each tube pairs were removed from the boiling water after immersing for the above time periods and replaced immediately in the ice bath. The extracts were then centrifuged at 3500 rev/min for 5 minutes and the clear supernatant solutions were directly used for electrophoresis.

The separation of eye lens proteins were electrophoretically processed as described in Chapter I.

RESULTS

The electrophoretic patterns of eye lens proteins are characterized by the number of protein fractions, mobility, width and staining intensity of the bands.

Whole eye lens proteins:

Ophiocephalus punctatus (Table 11 and Fig. 10):

Pattern A is produced from the extracts of unheated eye lens proteins. It consists of twelve fractions and has been described in Chapter II (page 40 & 41).

Patterns B & C are produced from the lens extracts which were heated for 1 sec and 3 sec respectively. Eight bands are detected in both the patterns, of which six bands are on the cathodal side and two bands are towards the anode. The pattern C is unlike B in that on the anodal side the fractions (b & c) are less intensely stained. Three farthest migrating protein components (d, e & f) and one slow migrating protein component (e) on the anodal side have been denatured by heating.

Pattern D is of protein from the lens extract which was heated for 5 sec. Six fractions are found, only one band (c) is on the anodal side which is very lightly stained with poor resolution. This pattern is unlike of patterns B & C in that the closest migrating fractions (b on the anodal side and 1 on the cathodal side) of the point of sample application are absent. On the cathodal side other bands are similar to patterns B & C.

Pattern E is of protein extracts heated for 8 sec and shows only five cathodic migrating protein components, but no bands are found on the anodal side. Unlike pattern D, band (m) is less intensely stained and other bands are well stained similar to previously described fractions of pattern D.

Pattern F is produced by the extracts heated for 14 sec and consists of three fractions only. Fraction (k) is lightly stained and other two farthest bands are intensely stained.

No remarkable differences are observed among the patterns of G & H, produced from the lens extracts heated for 18 and 24 sec respectively. Though two fractions (n & o) are found in both the patterns but in pattern H, fraction(n)is less stained than the pattern G.

Ophicephalus striatus:

The protein patterns produced from the unheated and heated lens extracts of _O. striatus_ have been represented in Table 12 and Fig. 11.

Pattern A is of protein from the unheated lens extracts. Ten fractions are obtained which have been described in Chapter II (page 41&42).

Patterns B & C are produced from the lens extracts which were heated for 1 sec and 3 sec respectively. Three bands are found on the anodal side and five on the cathodal side. The farthest migrating protein band (f) on the anodal side and band (i) closest to the point of sample application on the cathodal side are absent. The farthest migrating bands (n & o) on cathodal side are in contact and intensely stained.

Pattern D is of protein from 5 sec heated lens extracts and shows five fractions. Only one band is found on the anodal side which is less stained. On the anodal side the disappearance of the fractions (b & d) and unequal width of the fraction(e) differs from the patterns B & C. On the cathodal side more or less all the fractions are similar to patterns B & C except the disappearance of i band.

Cathodally migrating three fractions have been observed in the pattern E, which is produced from the lens extracts heated for 8 sec. No anodic migrating protein fraction is found in this pattern.

Pattern F is produced from the lens extracts heated for 14 sec and consists of two fractions (n & o) on the cathodal side which are well stained.

No observable differences are found among the patterns G & H, which are produced from the lens extracts heated for 18 and 24 sec respectively. Two farthest migrating protein fractions (n & o) are found but very lightly stained.

<u>Nuclear lens protein:</u>

<u>Ophiocephalus punctatus</u>: (Table 13 and Fig. 12).

Pattern A is produced from the unheated nuclear lens extracts of <u>O. punctatus</u> and consists of four fractions, similar to patterns A (Fig. 9, Chapter IV).

Pattern B is of protein from a nuclear lens extract which was heated for 14 sec and shows three fractions. All the fractions are intensely stained except unequal width of band k.

Pattern C is produced from the nuclear lens extract which was heated for 18 sec. Two farthest migrating components (n & o) are detected which are intensely stained.

Ophicephalus striatus: (Table 13 and Fig. 12).

Pattern A_1 is produced from the lens extracts of _O. striatus_ which was not heated and four bands are observed toward the cathode. The farthest migrating protein bands (n & o) are intensely stained but the band(k) is broad and well stained. Band (m) is very lightly stained with very poor resolution.

Pattern B_1 is produced from the nuclear lens extracts, which was heated for 8 sec. Band(k) is absent and other fractions are as those of pattern A_1.

Pattern C_1 consists of two fractions which is produced from the heated extracts for 14 sec. In this pattern fraction(m) has disappeared. The farthest migrating two protein fractions (n & o) are lightly stained with poor resolution.

DISCUSSION

The denaturation of whole eye lens proteins were electrophoretically observed in _O. punctatus_ and _O. striatus_ and different patterns were found for heated lens extracts of different time intervals. The molecular configuration of proteins was found to be denatured by heat in a specific way. Similar observations have been made by Polard, 1959, Tanford, 1961 and Wood, 1968.

Interspecific differences are clearly demonstrated in nuclear lens proteins between _O. punctatus_ and _O. striatus_. A pair of far migrating protein bands toward the cathode are of similar mobility. Less obvious differences are observed only in staining intensity. Other differences are; 1. j band is present in _O. punctatus_ but absent in _O. striatus_. 2. a band is absent in _O. punctatus_ whereas present in _O. striatus_.

The protein extracts of whole eye lens are found to be effected greatly by exposing to heat whereas no effect was noted for nuclear lens proteins of _O. punctatus_ and _O. striatus_ until exposed for 14 seconds and 8 seconds respectively. The above observations indicate a higher thermostability in the nuclear lens proteins of _O. punctatus_ than in _O. striatus_.

A high thermostability of nuclear lens proteins has been recorded in bigeye tuna than the yellowfin tuna (Smith, 1970). Other studies on thermostability differences of actomyein, adenosine triphosphate, hemoglobins, collagens and enzymes have also been demonstrated among the closely related animal species (Ushakov, 1964, 1967).

The present study also indicates a less thermostability in whole eye lens proteins than that of nuclear lens proteins. The nuclear lens proteins are highly thermostable and resist denaturation by heat.

Comparatively higher thermostability of nuclear lens proteins and whole lens proteins is demonstrated by proteins of higher electrophoretic mobility on the cathodal side. Only a few changes in the protein patterns are affected on the cathodal side by exposing the extracts to heat for different time intervals. The farthest migrating proteins of high molecular weight are in greater persistence than the slow migrating proteins of low molecular weight. This confirms the findings of Smith (1966a) on cortical lens proteins of channel rockfish (*Sebastolobus alascanus*); nuclear lens proteins from yellowfin and bigeye tuna (Smith, 1970); aldolases from muscle of *Rana temporaria* and of *Rana ridibunda* (Ushakov, 1967), from whole eye lens proteins of birds (Sibley and Brush, 1967) and esterases from bovine liver (Ecobichon, 1966).

In whole eye lens proteins of both the species of *Ophiocephalus* it has been found that no anodic migrating proteins are found after heating the extract for 6 sec. The protein patterns of whole eye lens are affected as soon as they are exposed to heat whereas the nuclear lens proteins of *O. punctatum* and *O. striatum* denature after an exposure of 14 sec and 8 sec respectively. The anodic migrating proteins are found to be denatured rapidly than the cathodic migrating proteins. As there is no anodic migrating proteins in nuclear lens of these two species, therefore, anodic proteins are all cortical proteins which are extremely sensitive to heat and denature rapidly rather than nuclear lens proteins.

Although the heated proteins denaturate and the degree of denaturation depends upon the time of exposure to heat, the interspecies differences could be discerned from electrophoretic patterns by differential mobility of remaining proteins. In whole eye lens, four bands in *O. punctatus* and two bands in *O. striatus* disappeared after heating for one sec. After five sec of heating. Six bands (c on the anodal side and j,k,m,n & o on the cathodal side) were present in *O. punctatus* whereas five bands (c on the anodal side and k,m,n & o on the cathodal side) were present in *O. striatus*.

Smaller species differences were observed after 18 or 24 sec of heating, where two farthest migrating protein fractions (n & o) were present in both the species, differing only in staining intensity.

SUMMARY

Proteins extracted from whole eye lenses and nuclear lenses of two closely related species of *Ophiocephalus punctatus* and *Ophiocephalus striatus* were heated for different time intervals (sec) and then electrophoretically separated using starch gel as supporting medium.

In both of these species of *Ophiocephalus*, proteins of whole eye lens denatured more rapidly than the nuclear lens

proteins, indicating a high thermostability of nuclear lens proteins.

Electrophoretic separation of whole and nuclear lens proteins of both the species revealed similar electrophoretic patterns, as expected from closely related species. Protein extracts heated for different time intervals showed increasing denaturation of proteins. The proteins migration on anodal side were more effected than on the cathodal side. Greater thermostability differences were found in slow migrating proteins.

TABLE 11. SUMMARY OF THE PROTEIN ZONES IN THE WHOLE EYE LENS ELECTROPHEROGRAM OF O. PUNCTATUS PRODUCED BY UNHEATED EXTRACTS (A) AND HEATED EXTRACTS FOR THE FOLLOWING TIME PERIODS (sec): 1 (B), 3 (C), 5 (D), 8 (E), 14 (F), 18 (G) AND 24 (H). VERTICAL LINE INDICATES THE ORIGIN. RELATIVE INTENSITIES AND MOBILITIES OF BANDS ARE NOT CONSIDERED HERE.

Patterns	ALPHABETICAL POSITIONS OF PROTEIN ZONES												
	Cathode							Anode					
	o	n	m	l	k	j	i	a	b	c	d	e	f
A	+	+	+	+	+	+	-	+	+	+	+	+	+
B	+	+	+	+	+	+	-	-	+	+	-	-	-
C	+	+	+	+	+	+	-	-	+	+	-	-	-
D	+	+	+	-	+	+	-	-	-	+	-	-	-
E	+	+	+	-	+	+	-	-	-	-	-	-	-
F	+	+	-	-	+	-	-	-	-	-	-	-	-
G	+	+	-	-	-	-	-	-	-	-	-	-	-
H	+	+	-	-	-	-	-	-	-	-	-	-	-

+ = Presence of protein zone

- = Absence of protein zone

TABLE 12. SUMMARY OF THE PROTEIN ZONES IN THE WHOLE EYE LENS ELECTROPHEROGRAM OF O. STRIATUS. PATTERN PRODUCED BY UNHEATED EXTRACTS (A) AND HEATED EXTRACTS FOR THE FOLLOWING TIME PERIODS (sec): 1 (B), 3 (C), 5 (D), 8 (E), 14 (F), 18 (G) AND 24 (H). VERTICAL LINE MARKS THE ORIGIN. RELATIVE INTENSITIES AND MOBILITIES OF BANDS ARE NOT CONSIDERED HERE.

Patterns	ALPHABETICAL POSITION OF PROTEIN ZONES												
	Cathode							Anode					
	o	n	m	l	k	j	i	a	b	c	d	e	f
A	+	+	+	+	+	-	+	-	+	+	+	-	+
B	+	+	+	+	+	-	-	-	+	+	+	-	-
C	+	+	+	+	+	-	-	-	+	+	+	-	-
D	+	+	+	-	+	-	-	-	-	+	-	-	-
E	+	+	+	-	-	-	-	-	-	-	-	-	-
F	+	+	-	-	-	-	-	-	-	-	-	-	-
G	+	+	-	-	-	-	-	-	-	-	-	-	-
H	+	+	-	-	-	-	-	-	-	-	-	-	-

+ = Presence of protein zones

- = Absence of protein zone

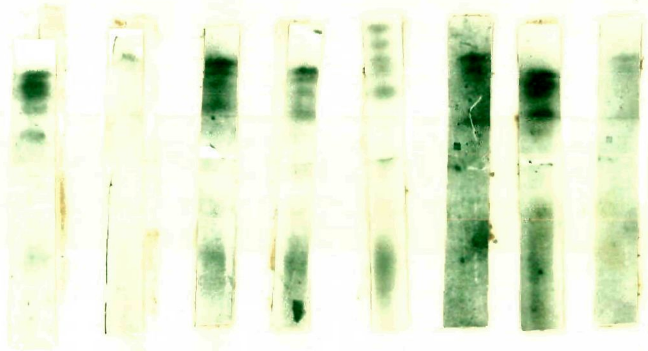
TABLE 13. SUMMARY OF THE PROTEIN ZONES APPEARING IN THE NUCLEAR LENS PROTEIN PATTERNS OF *O. PUNCTATUS* AND *O. STRIATUS*. PATTERN A WAS PRODUCED FROM UNHEATED, B & C WERE PRODUCED FROM HEATED LENS EXTRACTS FOR 14 sec AND 18 sec RESPECTIVELY FROM *O. PUNCTATUS*. PATTERN A_1 WAS PRODUCED FROM UNHEATED, B_1 & C_1 WERE PRODUCED FROM HEATED EXTRACTS FOR 8 AND 18 sec RESPECTIVELY FROM *O. STRIATUS*. VERTICAL LINE MARKS THE ORIGIN. RELATIVE INTENSITIES AND MOBILITIES OF BANDS ARE NOT CONSIDERED HERE.

Patterns	ALPHABETICAL POSITION OF PROTEIN ZONES												
	Cathode							Anode					
	o	n	m	l	k	j	i	a	b	c	d	e	f
A	+	+	-	-	+	+	-	-	-	-	-	-	-
B	+	+	-	-	+	-	-	-	-	-	-	-	-
C	+	+	-	-	-	-	-	-	-	-	-	-	-
A_1	+	+	+	-	+	-	-	-	-	-	-	-	-
B_1	+	+	+	-	-	-	-	-	-	-	-	-	-
C_1	+	+	-	-	-	-	-	-	-	-	-	-	-

+ = Presence of protein zones

- = Absence of protein zones

Fig. 10. Electrophoretic patterns of unheated and heated extracts of eye lens protein from *Ophicephalus punctatus*. Pattern A is of protein from an unheated extract and is the same as patterns of B,C,D,E,F,G and H from extracts heated for as long as (sec) 1,3,5,8,14,18 and 24 respectively.



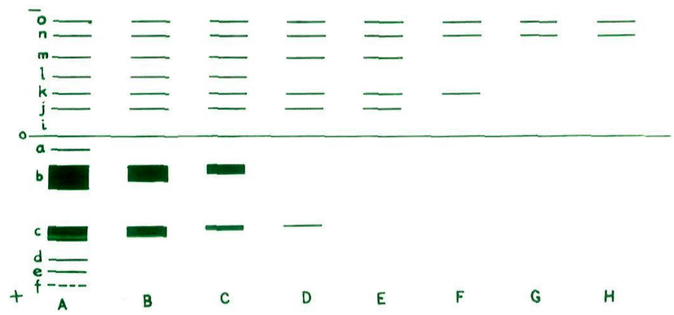


FIG 10

Fig. 11. Electrophoretic patterns of unheated and heated extracts of eye lens proteins from _Ophicephalus striatus_. Pattern A is of protein from an unheated extract and is the same as patterns of B,C,D,E,F,G & H from extracts heated for as long as (sec): 1,3,5,8,14, 18 and 24 respectively.

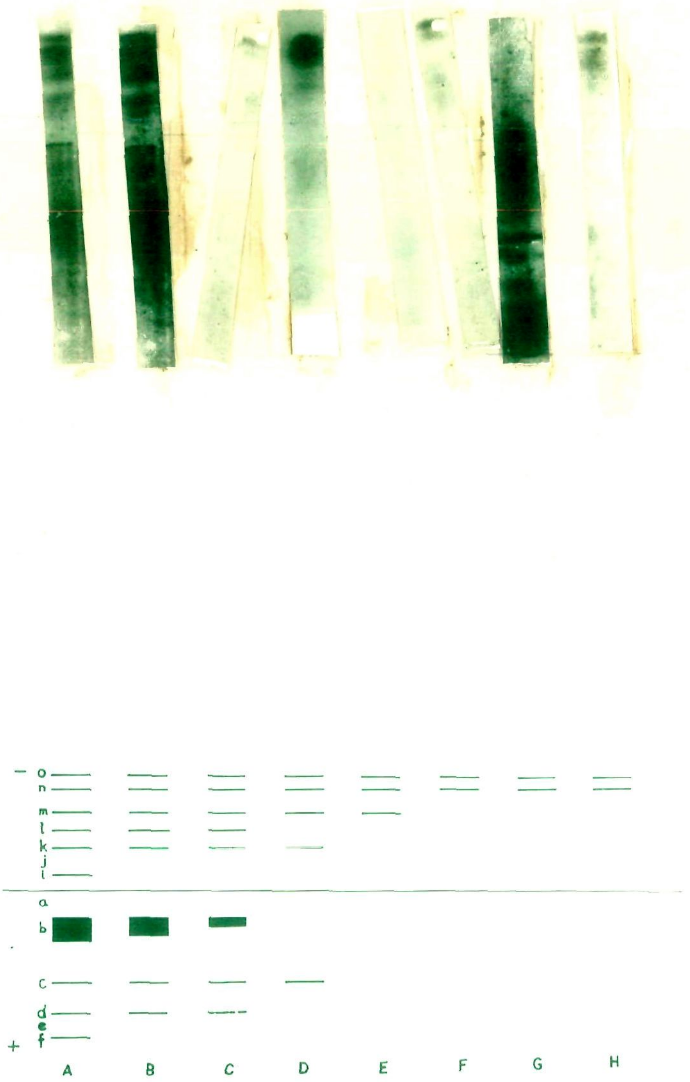


FIG 11

Fig. 12. Nuclear lens protein patterns of _Ophicephalus punctatus_ and _Ophicephalus striatus_. Patterns A, B & C were produced from unheated and heated extracts of 14 sec and 18 sec respectively from _O. punctatus_ and patterns A_1, B_1 & C_1 were produced from unheated and heated extracts of 8 and 10 sec respectively from _O. striatus_.

FIG 12

CHAPTER - VI

BLOOD PROTEINS OF SOME FRESHWATER FISHES OF ALIGARH

INTRODUCTION

The method of electrophoresis is widely used for separating serum proteins (Ribeiro et al., 1961; Tsuyuki and Roberts, 1966; Thurston, 1967, Hasnain et al., 1973b). In fishes as also in other vertebrates, the serum protein patterns observed are found to be species specific (Saito, 1957; Nyman, 1965). It provides an useful basis for taxonomic studies (Chandrasekhar, 1959; Nyman, 1965), as also a practical method for recognising subpopulations of a species (Marr, 1957; Brooke, 1964).

However, there are variations in

of the same fish species influenced by such factors as the degree of sexual maturity (Vanstone and Ho, 1961), fasting (Drilhon et al., 1958) and seasonal changes (Saito, 1957). Temperature (Meisure and Hickman, 1962) and certain pathological conditions (Flemming, 1958) also affect such changes. Genetical differences among populations of different vertebrates based on serum transferrin characteristics have also been established (Moller, 1966; Moller and Naevdal, 1966; Barrett and Tsuyuki, 1967).

Besides protein fractions, characteristics enzymes as serum esterases in fishes and other vertebrates have also been used to diagnose populations, stocks, races and hybrids (Nyman, 1967a; Nyman and Westin, 1969; Chen and Tsuyuki, 1970). These provide clues in recognising evolutionary relationship.

Realising the paucity of such information on Indian freshwater fishes (Chandrasekhar, 1959), a detailed investigation on the protein make up of the blood serum of 13 freshwater common species of Aligarh waters was undertaken. In addition, lipoprotein, transferrin and serum esterases of few species have also been studied electrophoretically.

MATERIALS AND METHODS

The fishes used for the analysis of serum proteins, lipoprotein, transferrin and serum esterases were obtained from different water resources (Table 14).

Buffer systems, experimental procedures for starch gel electrophoresis and staining methods are described in Chapter I.

RESULTS

The normal serum protein patterns of carps, *L. rohita*, *C. mrigala* and *C. catla* are represented in Fig. 13.

Three different electrophoretic patterns (A, B, & C) are observed in *L. rohita* (Fig. 13A,B, & C). Seven fractions are found in pattern A (River Kali sample) and all are on the anodal side. Two fractions (a and b) nearest to the point of origin are very lightly stained, other two protein zones (c & e) are moderately stained, while band(g) is found to be the broadest and darkest among all the fractions. The farthest migrating two protein fractions (i & k) are intensely stained.

Pattern B is also produced from river Kali fishes and consists of eight protein zones (Fig. 13B). All the protein components are similar to pattern A, except the presence of an additional protein zone (d), which takes heavy stain.

Pattern C is produced by serum proteins of L. rohita collected from river Ganga and Panethi canal and reveals seven fractions, which are similar to first six protein fractions of pattern B. Fractions (i & k) of A & B patterns are absent, but one protein zone (j) is present between the two fractions (i & k) of patterns A and B.

Two patterns (D & E) are produced by the serum proteins of C. mrigala (Fig. 13D & E). Patter D is produced from river Kali and consists of six fractions. Fraction (a) is broad but lightly stained whereas three fractions (c, d & e) exhibit high staining reaction and are similar to fractions (c, d & e) of patterns B & C (Fig. 13) of L. rohita. Fraction (g) is broad and darkly stained and the farthest migrating fraction (j) is moderately stained.

Pattern E of *C. mrigala*, obtained from Panethi canal, consists of seven fractions and is different from the pattern D (Fig. 13D). The protein zones (d and j) are absent, while farthest migrating two protein zones (i & k) are similar to those of patterns (A & B) of *L. rohita*. A great similarity is observed with the pattern A of *L. rohita*, it differs only in staining intensity.

Three different patterns (F, G & H) are observed in the electropherogram of *C. catla* (Fig. 13F, G & H), of which the pattern H is produced by immature fish.

Seven fractions are obtained in the serum protein patterns of fish collected from lake Sheikha (Fig. 13F). All the fractions are more or less intensely stained except the band g, which is broad and darkly stained.

Pattern G (Fig. 13G) which is produced from the serum proteins of river Kali, consists of nine fractions. The band b of pattern F is absent in this pattern, while three additional protein fractions (f, h & k) are observed. Fraction (e) is lightly stained in comparison to the fraction (e) of pattern F.

Serum proteins of *M. gitu* produced two distinct patterns (Fig. 14A & B). Pattern A is observed from the fishes of river Kali, while pattern B produced by fishes of lake Sheikha. Pattern A reveals eight fractions, while five fractions are found in pattern B. There occurs some fusion of protein fractions in the latter case.

Seven fractions are observed in the electrophoretic pattern of R. vacha (Fig. 14C). Population differences are not seen in this fish.

The serum protein pattern of C. bimaculatus consists of nine fractions (Fig. 14D). Farthest migrating two fractions are broad, darkly stained and close together. Remaining fractions also show strong staining reaction.

The serum protein of H. fossilis is found to contain 9 fractions (Fig. 14E). Intraspecific differences in the population of river Kali are not established.

Two patterns (I & J) are produced by A. testudineus. Pattern I (Fig. 14I) is produced by the fishes of Burdwan (West Bengal) and consists of nine fractions, whereas eight fractions are found in pattern J (Fig. 14 J) obtained from the population of Aligarh. One slow migrating fraction (e) and one farthest migrating fraction (m) of pattern J are lightly stained than that of pattern I. Besides, one farthest migrating fraction (n) is absent in pattern I. Remaining fractions are similar in both patterns.

Three different serum protein patterns (A, B & C) are observed for O. punctatus (Fig. 15A, B & C). Pattern A produced by lake Sheikha fishes consists of seven fractions. Pattern B is also produced from the same population and reveals nine fractions with an addition of two bands (b and h) while pattern C (Fig. 15C) is produced from river Kali and has only six fractions.

Ten individuals of O. gachua produced identical patterns and ten protein zones are detected (Fig. 15-D).

Serum protein pattern of O. striatus consists of eight fractions (Fig. 15E). Individual differences are not found in the fishes of any population.

For O. marulius two protein patterns (F & G) are found belonging to two different populations. The pattern produced by river Kali fishes (Fig. 15F) is quite different from that of lake Sheikha fishes (Fig. 15G). Intrapopulation differences are not detected.

Influence of sex and maturity:

In C. batrachus ten fractions are obtained for males (Fig. 14E) and eleven for females (Fig. 14F). Fraction (g) also varies in two sexes in regard to staining intensity.

In C. catla pattern H is produced by immature fish. Seven fractions are found. A clear difference of protein patterns is found between it (Fig. 13H) and other patterns (Fig. 13F & G) of C. catla.

Effect of starvation on serum proteins of H. fossilis:

50 mature individuals of H. fossilis were starved for three months. A sample of 5 fishes was analysed after every 15 days of intervals.

In starving fishes no significant change is noted in the first month. In the second month of starvation the staining intensity of fraction (k) gradually decreases. After 75 days of starvation two fractions (f & j) disappear (Fig. 14H). The fraction (k) remains as broad as that of normal serum protein but less dark. Thereafter no change in the characteristics of serum proteins is observed.

Lipoprotein:

Three lipoprotein fractions are separated in the sera of O. punctatus and C. batrachus (Fig. 16A & B). In both sexes same number of lipoprotein fractions are recorded throughout the year although the middle fraction is darkly stained in prespawning females.

Transferrin:

Electrophoretic separation of transferrins could be attempted in case of only O. punctatus and C. batrachus. In both species two intensely staining green bands are detected but there are differences in mobility of the bands (Fig. 16C, C_1 & D). Intraspecific variations are not observed in transferrin fractions but the general pattern of C. batrachus is found to vary.

Serum esterases:

The sera of C. punctatus, C. gachua, C. striatus and C. marulius are examined for non-specific esterases. The normal esterase pattern of each species is one molecular form, i.e., only one band in each species (Fig. 17). The esterases of these species differ from each other in mobility.

DISCUSSION

A comparison of the serum protein electropherograms of 13 freshwater fish species revealed a remarkable parallelism between the electrophoretic patterns of closely related species. The present study indicates that if the two species are closely related, the electrophoretic patterns are found to be similar or identical within the specific limits of the particular group of proteins. This observation is consistent with other studies in various tissue proteins (Nyman, 1967b; Nyman and Westin, 1969; Tsuyuki et al., 1965, 1968; Cowan, 1972).

Intraspecific variation occurred in most of the species in the serum protein patterns. A few bands are, however, common in the species of the same genus and may be used as characters for species discrimination.

Electrophoretic patterns of three major carps, *L. rohita*, *C. catla* and *C. mrigala* are quite different from each other, though similarities exist in some common bands (Fig. 13). Chandrasekhar (1959) also reported similarities in the serum protein patterns of five major carps, by means of agar gel electrophoresis. He detected only five components in each species whereas more than five fractions are observed in the serum protein electropherograms of the three major carps examined presently. It becomes quite evident that starch gel electrophoresis separated more components and give better result than agar gel. Stansby (1954) has reported a similar observation of interspecies variations in his study of the freshwater fishes of United States.

Intraspecific differences in *L. rohita* are found to be rather slight. The population of river Kali produced two patterns. The intraspecies variation in the relative concentration of albumin reported for fish apparently is influenced by environment, nutrition level and other physiological factors. It has also been reported that the concentration of albumin is reduced or absent in freshwater bony fishes (Drilhon, 1953; Magnin, 1958; Sulya *et al.*, 1961) but higher in marine species (Woods and Engle, 1957; Engle *et al.*, 1958). Thus differences between the two populations of river Kali and Panethi may not be hereditary, but due to some other factors, physiological, nutritional or environmental. On the other hand, variations in the serum proteins of river Kali may be due to hereditary differences.

The *Catla catla* population in lake Sheikha was quite different from that of river Kali as evidenced by their serum protein structure. Intraspecific differences were clearly observed between these two populations. Differences in the band number and mobility appear to be a direct proof of their belonging to different gene pools. Nyman (1967b) also reported the variation of serum protein patterns between two salmon populations (blego salmon of Norway and Swedish salmon).

Significant differences were also noted in the electrophoretic patterns of *W. attu*. Diffusion of bands in pattern B, which differs from pattern A, may be caused by many factors.

Pattern variations were not demonstrated in *E. vacha* and also in *C. bimaculatus*.

The serum protein patterns of *A. testudineus* from two populations also varied but only in staining intensity. Nyman (1967b) reported similar differences in various populations of Atlantic salmon.

Variations in serum protein patterns of *O. punctatus* are also well marked. Slight intrapopulation differences were observed in lake Sheikha.

No intraspecific differences were recorded in the serum proteins of *C. gachua*. It might be due to sampling of few specimens.

The serum protein pattern of *C. striatus* does not exhibit any variations in the population of river Kali and Sheikha ditches, but the sera of *C. marulius* showed variations between these two populations.

Influence of sex and maturity:

Only slight differences were noted between the male and female serum protein patterns in *C. batrachus*. Similar observations have been made by Hall *et al.*, 1961; Tsuyuki and Roberts, 1966 and Aida *et al.*, 1973.

Analysis of serum protein of immature and mature fishes of *C. catla* produced different patterns whereas identical patterns were obtained for fishes of the same maturity.

Effect of starvation:

Starved *H. fossilis* did not show any variation in its serum protein in the first month of starvation. In the second month the concentration of albumin fraction decreased. Significant changes were observed in the third month when two bands were found to disappear. Thereafter no changes occurred. The electrophoretic pattern of blood proteins of *Salmo trutta* has been reported to change after 30 days of starvation (Drilhon,

1954) and a period of 6 months was required to change the pattern of *Cyprinus carpio*. The concentration of blood serum protein in *Scyllium canicula* changed within 15 days while this period was not sufficient to affect such change in *Anguilla anguilla* (Cordier *et al*., 1957).

No remarkable difference was observed between the pattern of fresh and frozen sample. Most of the serum samples were analysed within a week. Tsuyuki and Roberts (1966) reported that prolonged frozen storage (-30°C) of salmon plasma did not affect the patterns. Other workers have also stored the fish serum at different temperatures and reported no change of patterns drastically (Sanders, 1964; Thomas and McCrimmon, 1964).

Lipoprotein:

Three lipoprotein fractions were recorded in *C. batrachus* and *O. punctatus*, but with different mobilities. The middle fraction was intensely stained in prespawning female fish. This observation is in agreement with Aida *et al*. (1973) who reported one of the fractions attained the highest value before spawning.

Transferrin:

The serum transferrins did not show any polymorphism and consequently, no intraspecific variations were recorded in the intensely stained green bands. However different general protein patterns were noted in *C. batrachus*. Nyman and Westin (1969) also did not find any polymorphism in the serum transferrin of *Aronocephalus bubalis*. On the other hand, transferrin polymorphism has been reported in fishes by many investigators (Moller, 1966, Moller and Naevdal, 1966; Barrett and Tsuyuki, 1967, Koehn and Johnson, 1967; Fujino and Kang, 1968).

Esterase:

Serum esterase pattern has been reported to be a good indicators of species and family (Nyman, 1966a). This also holds true to a great extent with the species of *Ophicephalus*. The normal esterase pattern of each species of *Ophicephalus* is monomorphic i.e., one band in each species. Species differences are clearly observed because of different mobilities of esterase bands. No variation whatsoever has been found, all populations showing the same band. Monomorphic esterase pattern has also been reported in Salmon (Nyman, 1966a) and Cottid species (Nyman and Westin, 1969). Intraspecific variations in serum esterases of fish has also been reported (Koehn and Rasmussen, 1967; Nyman and Westin, 1968), and are due to the action of autosomal codominant allelic genes.

SUMMARY

The blood serum proteins of 13 species and few species for lipoproteins, transferrins and esterases were investigated by starch gel electrophoresis in combination with histochemical

staining methods. Intraspecific differences were observed in most of the species. These changes may be due to hereditary differences or inherent to size, rate of growth, environments and feeding habits. Examples of species specificity, ontogenetic variation, geographic variation and various polymorphisms are described, together with problems concerning the segregation of proteins.

Differences in protein patterns between mature and immature fishes of Catla catla were observed. The males and females produced similar patterns except C. batrachus. The starvation affected the proteins structure and the maximum change was effected after 75 days of starvation.

In prespawning and spawning females one of the three lipoproteins band stained intensely. No polymorphism was shown by transferrins.

Monomorphic serum esterase patterns were observed in four species of Ophiocephalus. No observable variation was established. Serum esterase fractions of different mobility in each species were found to be very useful for taxonomic purposes.

TABLE 14. NAMES, SEX, NUMBER OF INDIVIDUALS, SIZE RANGE AND PLACES OF CATCH USED FOR SERUM PROTEIN STUDIES.

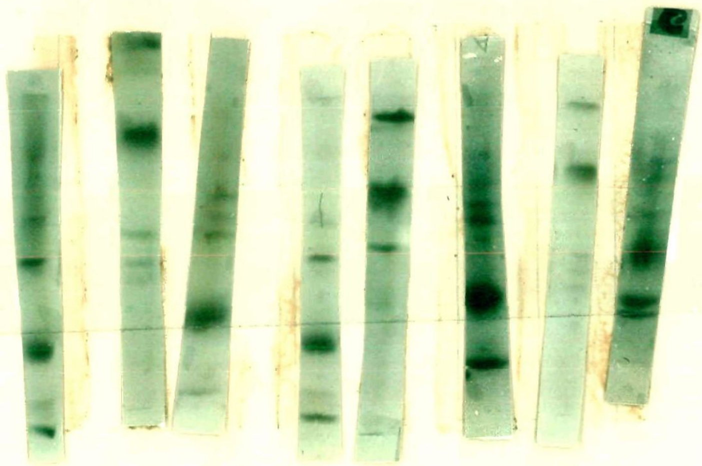
Species	Sex	Number of individuals	Size range (cm)	Locality
Labeo rohita	Male	35	20 - 30	River Kali, Aligarh
	Female	56	20 - 30	
	Male	110	22 - 28	River Ganga, Aligarh
	Female	25	22 - 28	
	Male	19	20 - 28	Panethi canal, Aligarh
	Female	35	20 - 28	
Cirrhina mrigala	Male	19	26 - 32	River Kali, Aligarh
	Female	32	26 - 32	
	Male	28	30 - 39	Panethi canal, Aligarh
	Female	39	30 - 39	
Catla catla	Male	28	26 - 30	Lake Sheikha, , Aligarh
	Female	31	26 - 30	
	Male	13	8 - 29	River Kali, Aligarh
	Female	32	8 - 29	
Wallagonia attu	Male	28	28 - 30	River Kali, Aligarh
	Female	36	28 - 30	
	Male	29	30 - 36	Lake Sheikha, , Aligarh
	Female	24	30 - 36	
Eutropiichthys vacha	Male	20	9 - 12	River Kali, Aligarh
	Female	35	9 - 12	
Callichrous bimaculatus	Male	150	15 - 22	River Kali, and Panethi canal, Aligarh
	Female	130	15 - 22	
Clarias batrachus	Male	50	22 - 25	River Kali, Aligarh
	Female	75	22 - 25	
	Male	75	20 - 28	Local market, Aligarh
	Female	59	20 - 28	

CONTINUED ....

TABLE 14 (CONTINUED)

Species	Sex	Number of individuals	Size range (cm)	Locality
Heteropneustes fossilis	Male	90	22 - 26	River Kali
	Female	126	22 - 26	
Anabas testudineus	Male	10	6 - 9	Local market, Aligarh and
	Female	50	6 - 9	Local market, Burdwan, West Bengal
Ophiocephalus punctatus	Male	125	11 - 18	Lake Sheikha, , Aligarh
	Female	225	11 - 18	
	Male	95	10 - 19	River Kali, Aligarh
	Female	119	10 - 19	
Ophiocephalus gachua	Male	3	12 - 18	Local market, Aligarh
	Female	7	12 - 18	
Ophiocephalus striatus	Male	120	20 - 35	River Kali and Sheikha ditches, Aligarh
	Female	167	20 - 35	
Ophiocephalus marulius	Male	35	22 - 30	River Kali, Aligarh
	Female	22	22 - 30	
	Male	19	20 - 35	Lake Sheikha, , Aligarh
	Female	26	20 - 35	

Fig. 15. Serum protein electropherograms of *Labeo rohita* (A, B & C), *Cirrhina mrigala* (D & E), *Catla catla* (F & G) and pattern H was produced from an immature fish of *Catla catla*.



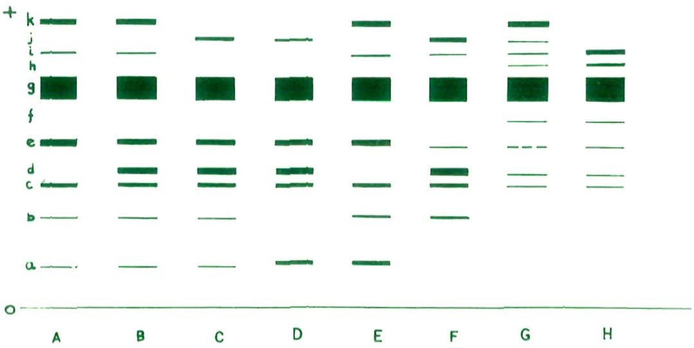
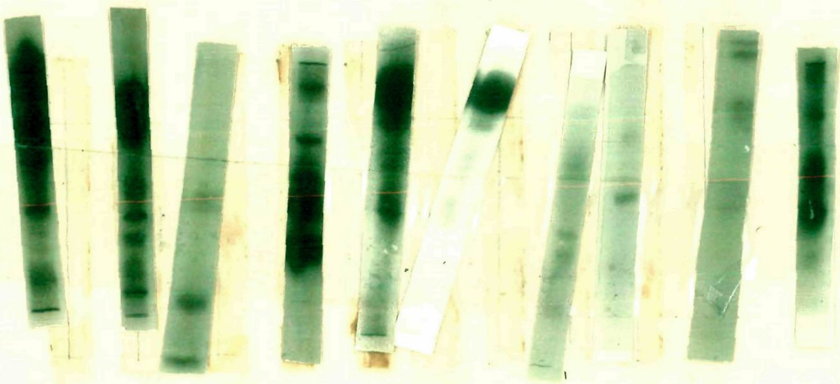


FIG. 13

Fig. 14. Serum protein electropherograms of *Wallagonia attu* (A & B), *Eutropiichthyes vacha* (C), *Callichrous bimaculatus* (D), *Clarias batrachus* (E ♂ & F ♀), *Heteropneu stes fossilis* (G normal pattern and H after 75 days of starvation) and *Anabas testudineus* (I & J).



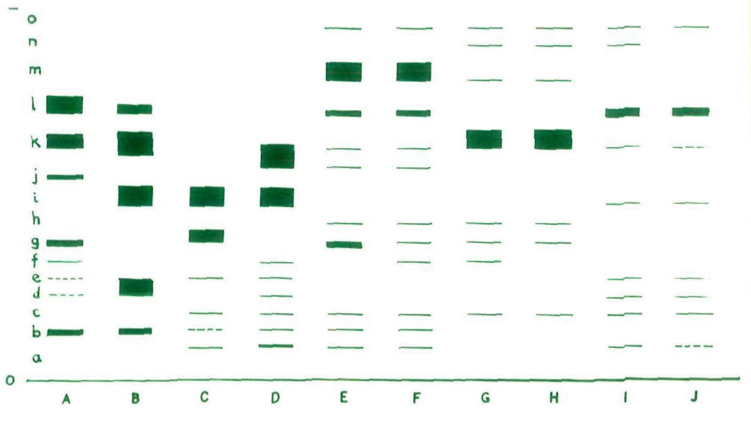
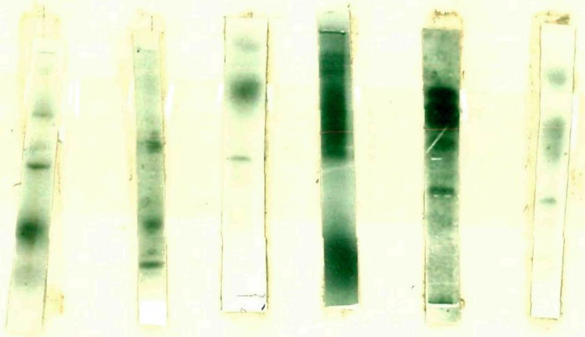


FIG. 14

Fig. 15. Serum protein patterns of *Ophiocephalus punctatus* (A & B from lake Sheikha, and C from river Kali), *O. gachua* (D), *O. striatus* (E), and *O. marulius* (F from river Kali and G from lake Sheikha).



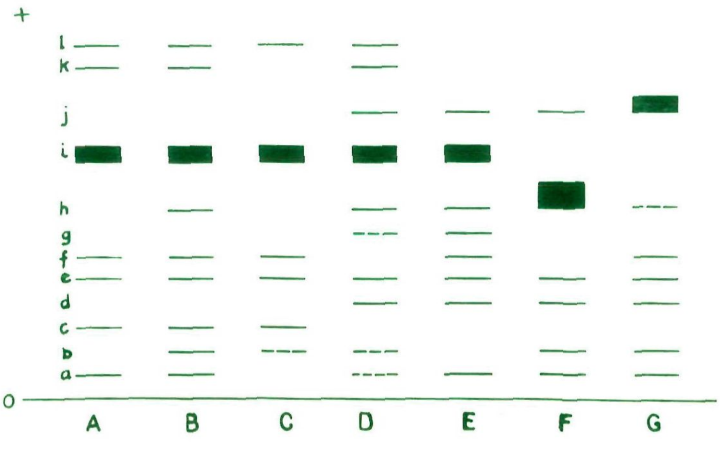


FIG 15

Fig. 16. Diagrammatic representation of normal lipoprotein electrophoretic patterns of (A) *Clarias batrachus*, (B) *Ophicephalus punctatus*. Nitroso-R stained serum protein patterns of *Clarias batrachus* (C & C_1), *Ophicephalus punctatus* (D). X = possible transferrin bands.

Fig. 17. Diagrammatic representation of the serum esterase patterns of *Ophicephalus punctatus* (A), *O. gachua* (B), *O. striatus* (C) and *O. marulius* (D).

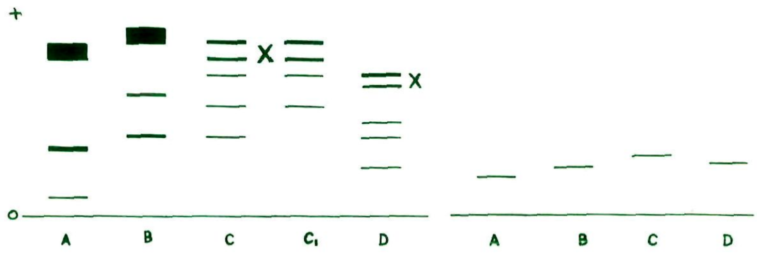


FIG. 16

FIG. 17

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